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Poster Abstracts
Protein Structure and Biology

PS.01: Optimisation of real-time QuIC

Lynne I. McGuire,[1] Graham Fairfoul,[1] Nigel E. Appleford,[2] Gary Mallinson,[2] Robert G. Will,[1] Richard S. Knight[1] and Alison J. Green[1]

[1]NCJDSU; University of Edinburgh; Edinburgh, UK; [2]Bristol Institute for Transfusion Sciences; Bristol, UK

The ability of PrP^{Sc} to convert PrP^{C} into protease-resistance isoforms has been exploited using a variety of techniques such as protein misfolding cyclic amplification (PMCA), quaking induced conversion (QuIC) and most recently, real-time quaking induced conversion (RT-QuIC).[1] These cell-free assays have enabled a better understanding of prion diseases and have facilitated the development of potential diagnostic tests for prion-related diseases.

The RT-QuIC technique exploits the ability of PrP^{Sc} in brain tissue or CSF to induce a recombinant PrP to change shape and aggregate over time. This aggregation is observed by the binding of Thioflavin T (ThT) in the reaction mixture to the aggregates causing a change in the ThT emission spectrum, which can be monitored in real time.

Studies have shown that CSF samples from hamsters inoculated with experimental scrapie and from patients with sCJD can be correctly identified using RT-QuIC.[1-3] At the National CJD Research and Surveillance unit (NCJDRSU) we have completed a retrospective study and are currently undertaking a prospective audit investigating the value of RT-QuIC in the diagnosis of sCJD.[3] During our studies various elements of the technique have been modified during the optimisation process of this potential sCJD CSF diagnostic tool such as: the volume of CSF; the source of recombinant PrP (rPrP); the size of construct (Full-length or Truncated); and the shaking time, speed and mechanism used, each can make a significant difference to the results obtained. This study looks at some of these variables and what the effect of altering them has on the results and the ultimate understanding of the technique.

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PS.02: Oxidation of methionine in PrP is dependent upon the oxidant and the amino acid two positions removed

Christopher J. Silva, Irina Dynin, Melissa L. Erickson, Colleen Hui and J. M. Carter

USDA; Albany, CA USA

Background/Introduction. Methionine oxidation has been shown both to be associated with prion formation and implicated in the inhibition of amyloid formation in model systems. This work is based on model systems where hydrogen peroxide was used as an oxidant.

Materials and Methods. We developed a sensitive mass spectrometry-based method to study the oxidation of a methionine at position 216 (Met216) in sheep PrP. Oxidation of Met216 (MetSO216) has been implicated in prion formation. In order to test the susceptibility of Met216 to oxidation by molecular oxygen, we prepared clones containing three polymorphisms of sheep PrP at position 218 (Ile, Val and Thr). The clones were grown in medium supplemented with either natural abundance NH4Cl or 15NH4Cl, in order to obtain isotopically labeled recombinant protein. The recombinant proteins were purified and then subjected to air oxidation.

Results and Conclusions. Our analysis showed that the proportion of MetSO216 was highly dependent upon the amino acid residue at position 218. The Met216 in the sheep rPrP protein containing 218Ile was more susceptible than the Met216 in the sheep rPrP with Val218. The Met216 was least oxidized when the protein contained Thr218. In model studies, the Met216 is resistant to oxidation when the oxidant is hydrogen peroxide. This indicates that the presence of Ile218 in sheep and elk PrP renders the Met216 intrinsically more susceptible to air oxidation. Our results also indicate that Val218 is likely to make the analogous methionine in the human form of PrP more susceptible to air oxidation.

PS.03: Size, not density of strain-specific prion particles determines their replication dynamics

Florent Lafferière, Philippe Tixador, Mohammed Moudjou, Jérôme Chapuis, Pierre Sibille, Laetitia Herzog, Fabienne Reine, Emilie Jaumain, Human Rezaei, Hubert Laude and Vincent Béringue

INRA; Jouy-en-Josas, France

Prions are proteinaceous infectious agents responsible of fatal neurodegenerative diseases in animals and humans. They are essentially composed of PrP^{Sc}, an aggregated, misfolded isoform of the ubiquitously expressed host-encoded prion protein (PrP^{C}). Stable, inheritable variations in PrP^{Sc} conformation are assumed to encode the phenotypically tangible prion strains diversity. However the respective contributions of PrP^{Sc} secondary, tertiary and quaternary structure to the strain biological information remain mostly unknown. Applying a sedimentation velocity fractionation technique to a panel of ovine prions strains, categorized as fast and slow according to their incubation time in ovine PrP transgenic mice, has led to the intriguing observation that the relationship between prion infectivity and PrP^{Sc} quaternary structure was not univocal. For the fast strains specifically, infectivity segregated from the bulk of proteinase-K resistant PrP^{Sc} and peaked markedly in the upper top fractions of the gradient.[1] Here we examined whether these properties were due to a small size or density. Running the gradients at the equilibrium revealed that the density profile of prion infectivity and PK-resistant PrP^{Sc} tended to overlap whatever the strain, fast or slow, and the solubilization conditions. This indicates that a small PrP^{Sc} aggregation size accounts for the low sedimentability of the fast strain most infectious component. We further show that its resistance to limited PK-proteolysis and its templating activity by protein misfolding cyclic amplification outclasses that of larger size PrP^{Sc} aggregates. Together the tight correlation between small size, conversion efficiency and duration of disease suggest that PrP^{Sc} quaternary structure is a determining factor of prion replication dynamics.

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PS.04: The cellular prion protein octarepeat region as a recognition motif for hemin binding

Efrosini Artikis, William E. Johnson, Jr., Thayumanasamy Somasundaram and Ewa A. Bienkiewicz,

Florida State University; Tallahasee, FL, USA

The prion protein exists in two forms. The first (PrP^{Sc}), is notoriously associated with irreversible neurodegeneration and fatal disease. In stark contrast, the normal, non-pathological, form of the prion protein (PrP^{c}, or cellular PrP) appears to encompass numerous critical cellular functions that include signal transduction, neuroprotection, and angiogenesis. Although the N-terminal domain of the cellular prion protein has been classified as intrinsically disordered, it appears to have elements of structural organization and serves as a recognition motif for binding of a number of ligands, including hemin. Importantly, PrP^{c} has been shown to be an important player in the response to vascular injury, with the cell death and damage due to stroke shown to be significantly more extensive in the absence of PrP^{c} . In vascular injury, one of the critical events is the release of toxic levels of free hemin that damage the surrounding tissue. Since PrP^{c} has been shown to bind hemin, it is likely that the cellular prion protein plays a role in neutralizing the toxic hemin during stroke. This study tests the hypothesis that the N-terminal PrP^{c} domain fragments can serve as effective scavengers of free hemin. By doing so, they could potentially serve as therapeutic agents in vascular injury events. Equally importantly, the relevance of the hemin/N-PrP^{c} interaction examined in this study may extend to normal, physiological conditions, and help us understand the role this complex may serve in maintenance of heme/hemin homeostasis. The focus of our multi-faceted biophysical analysis is the interaction of hemin with the 2-repeat fragment (OR2) of the PrP^{c} N-terminal domain. Employed methodologies include isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), fluorescence, circular dichroism (CD) spectroscopy, and X-ray crystallography. As this research revolves around key aspects of protein folding, self-association, and macromolecular recognition, it is also anticipated that the resulting findings will provide new insights into the fundamental knowledge involving largely unstructured, but functionally important proteins and their domains.

PS.05: Molecular interaction of the cellular and pathogenic PrP describes the species barrier in prion diseases

Lars Luers,[2] Oliver Bannach,[2] Detlev Riesner,[2] Dieter Willbold[1] and Eva Birkmann[1]

[1]Research Centre Jülich; Jülich, Germany; [2]Heinrich-Heine University; Düsseldorf; Düsseldorf, Germany

Transmission of prion diseases is not restricted to one species, but occurs also between different species. In some cases a species barrier can be observed which results in limited or even unsuccessful transmission. The mechanism behind interspecies transmissibility or species barriers is not completely understood so far. The hallmark of prion diseases is the conversion of the host-encoded prion protein (PrP^{C}) to its pathological isoform PrP^{Sc}, which is accompanied by PrP aggregation.

To analyze this process at a molecular level, we established an in vitro seeded fibrillation assay, in which recombinant PrP (recPrP) can be specifically seeded by natural prion seeds.1 Only

recPrP as substrate and NaPTA precipitated prions from brain tissue as seed are required. Seeding is observed by fibril specific binding of thioflavinT. This seeding activity with pre-purified components is a direct consequence of the "prion-protein-only" hypothesis. Thus any seeding activity is expected to be based on a direct interaction of prion specific seeds and pure recPrP. To analyze the phenomenon of prion transmissibility between certain species and species barriers between others, we combined recPrP from one species with prion seeds of another species. Intraspecies fibrillation was always taken as control. Our in vitro seeding results (ref. 2 and Luers L, et al. Submitted) are in complete agreement with epidemiological observations and in vivo studies of species barriers and interspecies transmissibilites for CJD, CWD, BSE and Scrapie.

Regarding the molecular mechanism of species barrier we show that the seeding activity is sufficient to explain the species barrier phenomenon in prion diseases. We conclude that the molecular interaction of PrP^{C} and prion seeds is decisive for the seeding activity and thus for the species barrier phenomenon, although it cannot be excluded, that cellular factors or processes within the living organism influence transmissibility between species as well.

Assuming that (1) the "protein-only" hypothesis is correct and (2) cellular factors do not determine the species barrier, we hypothesize that the species barrier is based on the ability of a prion seed to recruit PrP^{C} as substrate. Thus, we conclude that our in vitro assay determines this seeding ability and therefore correlates with observations made in vivo. Furthermore, this enables us to investigate or even predict uncertain or unknown species barriers within hours compared with time consuming and expensive bioassays in transgenic rodents. We therefore hypothesize that the molecular mechanism of the species barrier is based on the molecular interaction of PrP^{C} and PrP^{Sc}.

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PS.06: In-Silico PrP^{Sc} inhibitor screening of 60,000 commercially available compounds

Nataraj Sekar Pagadala,[1] Rolando Perez-Pineiro,[1] Trent C. Bjorndahl,[1] Ritesh Kaur Saini,[1] Carol Ladner,[1] Mark Berjanskii,[1] Li Li,[2] Ebrima Gibbs,[2] Neil Cashman[2] and David S. Wishart[1]

[1]University of Alberta; Edmonton, AB Canada; [2]University of British Columbia; Vancouver, BC Canada

It has been reported that ligands with the same predicted binding site to monomeric prion protein (PrP^{c}) exhibit widely variable stabilizing/destabilizing effects. Furthermore, a correlation was observed between the molecules affinity and its ability to reduce PrP^{Sc} titers in ex-vivo studies. Building off this work, we confirmed experimentally the binding site and further developed a pharmacophore model of the stabilizing molecular interactions. Interestingly, we found that the 2-aminothiazole anti-prion compounds docked within this pocket and satisfied many of the favorable stabilizing interactions. Looking for unique inhibitors of prion misfolding, we screened the MOE database of 60,000 compounds using this scaffold and identified 115 compounds with potential anti-prion effects including novel pyrimidine analogs. Six of the compounds were chosen from the list based on their structural diversity and further assessed for their binding affinities and their effectiveness at reducing PrP^{Sc} titers in ScN2a cells. All of which all proved effective. These results and the pharmacophore model are presented. This study demonstrates the usefulness of computational methods to do in-silico screening for potential anti-prion therapeutics with molecular stabilizing effects.

PS.07: Binding of epigallocatechin gallate to monomeric, oligomeric and fibril forms of the syrian hamster prion protein

Trent C. Bjorndahl,[1] Rolando Perez-Pineiro,[1] Ritesh Kaur Saini,[1] Carol Ladner,[1] Nataraj Sekar Pagadala,[1] Mark Berjanskii,[1] Li Li,[2] Ebrima Gibbs,[2] Neil Cashman[2] and David S. Wishart,[1]

[1]University of Alberta; Edmonton, AB Canada; [2]University of British Columbia; Vancouver, BC Canada

Epigallocatechin gallate (EGCG) is a, naturally occurring alkyloid found in green tea. It has been shown to bind with nanomolar affinity to the monomeric prion protein and induce conformational instability. A better understanding of the specific molecular interactions that impart this strong interaction and molecular rearrangement are of interest for the development of ligands that possess both high affinity yet impart stabilizing effects. Recently, it has been demonstrated that molecules with such characteristics reduce PrP^{Sc} titers in both in-vitro and ex-vivo experiments. To gain mechanistic and structural insights into EGCG's effect on the monomeric prion protein (PrP^{c}), we have undertaken a number of studies involving tryptophan florescence quenching, in-vitro ScN2a cell assays, dot blot assays, acid-native gel analysis, NMR spectroscopy and computational docking of EGCG and its analogs, myricetin and gallic acid. In addition to this work, we have determined that EGCG also binds to prion oligomers and fibrils. More importantly, fibrils pretreated with EGCG are rendered seed incompetent.

PS.08: Interaction of a diazirine modified curcumin analog with the β-oligomeric form of the Syrian hamster prion protein

Rolando Perez-Pineiro, Trent C. Bjorndahl, Mark Berjanskii, Carol Ladner, Nataraj Sekar Pagadala and David S. Wishart

University of Alberta; Edmonton, AB Canada

Current strategies for developing therapeutics for fibril associated disorders (Alzheimers, Parkinsons and Crutzfeld Jacob diseases) involve a couple mechanistic approaches: those that break up preformed oligomers or fibrils (PrP^{Sc}) thus aiding cellular clearance, and those that bind to and stabilize monomeric PrP^{c}, thus preventing unfolding and refolding events which result in fibrilar formation. Curcumin, a natural curcuminoid from the spice turmeric, is a compound that falls into the latter category. Although curcumin does not bind monomeric PrP^{c}, it does interact with and disrupts preformed prion oligomers and fribrils (PrP^{Sc}). Understanding of this structural interaction and mechanistic dissociation would benefit the development of therapeutics; however, technical limitations exist with current structural biology techniques that have prevented the direct observation of this molecular interaction. Thus, we have modified curcumin with a known photo active diazirine crosslinker, which allows covalent attachment. Mass spectroscopic analysis of the resulting enzymatic fragments following the cross linking of curcumin to prion oligomers has allowed inference of this interaction and also provides structural clues into exposed regions of the intact prion oligomer. Validation of the methodology has been achieved using bovine serum albumin, another known curcumin binder, is also presented.

PS.09: Antiprion properties of orally active chemical chaperones

Leonardo M. Cortez and Valerie L. Sim

Centre for Prions and Protein Folding Diseases; University of Alberta; Edmonton, AB, Canada

A common characteristic of all amyloidoses is the accumulation of insoluble aggregates of protein in the extracellular tissue, which cannot be broken down by enzymes. At the present there is no effective therapy for any of the amyloidoses, making the search for new treatment compounds a priority. Unfortunately, the molecular bases of aggregation, the mechanism of toxicity of these aggregates and the biological function of the proteins involved in these diseases remain unclear, making difficult the rational design of drugs for an effective treatment. However, the accumulation of abnormally folded proteins triggers endoplasmic reticulum (ER) stress, and this has been suggested to play a central role in the pathogenic mechanism of amyloidoses.

There is evidence for a role of ER stress in prion disease also, where the normal prion protein (PrP^{C}) is misfolded to become the disease-associated aggregated form (PrP^{Sc}). Cell cultures treated with prions show an increase of ER stress markers. In addition, the induction of ER stress by tunicamycin leads to a significant increase of PrP^{Sc}. Interestingly, the brains of patients succumbing to the human prion disease Creutzfeldt-Jakob disease (CJD) show increasing levels of ER chaperones, also implying an induction of ER stress. In this context, modulators of ER stress would provide a strategy for counteracting prion diseases.

Chemical chaperones are a group of small molecules known to non-selectively stabilize protein conformation, improve ER folding capacity and alleviate ER stress. Some chemical chaperones such as trimethylamine N-oxide (TMAO), dimethylsulfoxide (DMSO) and glycerol are thought to stabilize PrP^{C} conformation and have been suggested to be effective to prevent PrP^{Sc} formation in vitro. In this work, we describe the influence of orally active chemical chaperones on PrP^{C} stability, seeded aggregation with different prion strains, and their abilities to cure prion-infected cell culture models.

We assessed chaperones using a modified version of the RT-QuIC assay and three different mouse prion strains (RML, 22L and ME7). Inhibitory effects found in this assay were correlated with effects seen in scrapie-infected cell culture. Interestingly, these inhibitory effects were not correlated with any increase in PrP^{C} stability, as assessed by thermal denaturation circular dichroism.

PS.10: Assessing prion species barriers using RT-QuIC methodology

Kristen A. Davenport, Davin M. Henderson, Candace K. Mathiason and Edward A. Hoover

Colorado State University; Fort Collins, CO, USA

The major known factors influencing the propensity for trans-species prion transmission are the structural characteristics of the donor and recipient prion proteins. Studies of the effects of primary or tertiary prion protein structures on prion transmission have relied upon transgenic or outbred animal bioassays, making the influences of prion protein structure vs. host co-factors (e.g., cellular constituents, trafficking, and innate immune interactions) difficult to dissect. As an alternative strategy, we are using real-time quaking-induced conversion (RT-QuIC) to investigate the propensity for and the kinetics of trans-species prion conversion. RT-QuIC provides better-defined and more easily mutable conditions of seeded conversion to study the processes involved in trans-species prion conversion. Specifically, full-length recombinant PrP^{c} from bovine, feline, human and elk are employed as substrates to assay the conversion potential of prion positive and negative bovine BSE, feline CWD, feline FSE, and white tail deer CWD brain homogenates. To assess the effects of species barriers, we are comparing (1) time to detection of PrP^{c} conversion to β sheet fibrillar forms, which is detected when an increase in fluorescence results from the binding of ThT to PrP^{Sc}, (2) maximum fluorescence and (3) mathematically modeled reaction

rates for homologous vs. heterologous substrate/seed combinations. To date we have detected delayed conversion and altered kinetics between trans-species and homologous full-length conversion or conversion employing a truncated, promiscuous rPrP substrate (e.g., hamster rPrP), putatively indicative of relative species barriers. The results of these ongoing studies should provide insight into the structural determinants of prion species barriers that encompass cross-species transmission risks.

PS.11: Multistep pathway to stable misfolded structures observed in single prion protein dimers by force spectroscopy

Derek R. Dee,[1] Hao Yu,[1] Xia Liu,[1] Angela M. Brigley,[2] Iveta Sosova[2] and Michael T. Woodside[1,2]

[1]Deptartment of Physics; University of Alberta; Edmonton, AB Canada; [2]National Institute for Nanotechnology; National Research Council; Edmonton, AB Canada

The detailed mechanisms of protein misfolding and aggregation remain largely intractable, due to the complexity of possible interactions and folding pathways. One approach to this problem is to study the folding of minimal oligomers of aggregation-prone proteins, determining at high resolution how misfolding proceeds. Toward this end, we studied the folding of two PrP molecules linked end-to-end to form dimers. Using optical tweezers to unfold and refold individual PrP dimers, we found that they behave very differently from monomers. Remarkably, neither domain of the dimer ever formed the native structure and instead formed exclusively a non-native structure. In contrast to the monomer, which folded without intermediates, dimer folding proceeded through multiple partially-folded intermediates. From the force spectroscopy measurements, we mapped the pathway to the misfolded structure, finding that an intermediate that folds rapidly at high force initiated the misfolding. These results provide insight into the microscopic mechanisms of structural conversion in prion protein misfolding.

PS.12: Post-translational changes to PrP alter transmissible spongiform encephalopathy strain properties

Abigail B. Diack,[1] Enrico Cancellotti,[1] Sukhvir Mahal,[2] Robert Somerville,[1] Deborah Brown,[1] Pedro Piccardo,[1,3] Charles Weissmann[2] and Jean C. Manson[1]

[1]The Roslin Institute & R(D)SVS; Easter Bush, UK; [2] Department of Infectology; Scripps Florida; Jupiter, FL USA; [3] Center for Biologics Evaluation and Research; Food and Drug Administration; Rockville, MD USA

The agents responsible for TSEs, contain as a major component PrP^{Sc} an abnormal conformer of the host glycoprotein PrP^{C}. TSE agents are distinguished by differences in phenotypic properties in the host, which nevertheless can contain PrP^{Sc} with the same amino acid sequence. If PrP alone carries information defining strain properties, these must be encoded by post-translational events. Here we investigated whether the glycosylation status of host PrP affects TSE strain characteristics. We inoculated wild-type mice with three TSE strains (79A, ME7 and 301C) passaged through transgenic mice with PrP devoid of glycans at the first, second or both N-glycosylation sites. We compared the infectious properties of the emerging isolates with TSE strains passaged in wild-type mice by in vivo strain typing and by the Standard Scrapie Cell Assay in vitro. Strain-specific characteristics of the 79A TSE strain changed when PrP^{Sc} was devoid of one or both glycans, in some cases exhibiting the phenotypic properties of the 139A strain, which has been derived from 79A several times previously, or that of a novel strain phenotype. In contrast, ME7 and 301C were not affected by the lack of one or both glycans on the host PrP. These results show that TSE infectivity can replicate in mice with glycosylation-deficient PrP, but with TSE strain-specific responses. This demonstrates that the carbohydrate moieties are not essential to TSE replication or retention of strain specific properties. Indeed despite the partial or complete absence of the carbohydrate moieties on PrP, TSE strain properties were maintained, or in the case of 79A altered to 139A in a similar fashion to changes taking place in wild-type mice. In other cases strain properties changed and a new TSE agent phenotype emerged. These results demonstrate that the glycosylation status of the host PrP can affect the replication and selection of a TSE strain but that TSE strain properties are independent from host PrP.

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PS.13: Convergent evolution of mouse synthetic prion strains

Sina Ghaemmaghami,[1] David W. Colby,[2] Oanh Nguyen,[3] Stephen J. DeArmond[3] and Stanley B. Prusiner[3]

[1]University of Rochester; Rochester, NY USA; [2]University of Delaware; Newark, DE USA; [3]University of California, San Francisco; San Francisco, CA USA

Prion diseases are neurodegenerative disorders characterized by the aberrant folding of endogenous proteins into self-propagating pathogenic conformers. Prion disease can be initiated in animal models by inoculation with amyloid fibrils formed from bacterially derived recombinant prion protein. The synthetic prions that accumulate in infected organisms are typically structurally distinct from the amyloid preparations used to initiate their formation and change conformationally on repeated passage. To investigate the nature of synthetic prion transformation, we infected mice with a conformationally diverse set of amyloids and serially passaged the resulting prion strains. At each passage, we monitored changes in the biochemical and biological properties of

the adapting strain. The physicochemical properties of each synthetic prion strain gradually changed on serial propagation until attaining a common adapted state with shared physicochemical characteristics. These results indicate that synthetic prions can assume multiple intermediate conformations before converging into one conformation optimized for in vivo propagation.

PS.14: The PrP^C C1 fragments derived from ovine prnp alleles exhibit different abundance in brain and in vitro fibrillisation characteristics

Wilfred Goldmann, Lauren Campbell, Andrew C. Gill and Nora Hunter

The Roslin Institute & Royal (Dick) School of Veterinary Studies; University of Edinburgh; Easter Bush, UK

Expression of the cellular prion protein (PrP^C) is crucial for the development of prion diseases. Therefore, resistance to prion diseases can result from reduced availability of the prion protein or from amino acid changes in the prion protein sequence. We propose here that increased production of a natural PrP α-cleavage fragment, C1, is also associated with resistance to disease. Our data show that steady-state levels of the C1 fragment relative to total PrP^C were 1.8 times higher in the cortex of ARR homozygous sheep (RR171), associated with resistance to disease, compared with disease-susceptible ARQ homozygous sheep (QQ171). Unexpectedly, only the C1 fragment derived from the ARR allele inhibited in-vitro fibrillisation of other allelic PrP^C variants. We hypothesis that the increased α-cleavage of ovine ARR PrP^C contributes to a dominant negative effect of this polymorphism on disease susceptibility. Furthermore, the RR171 samples were five times less likely to show PrP^C β-cleavage product C2 than QQ171 genotypes ($p \leq 0.01$). This significant reduction in C2 in sheep of the resistant genotype may add to the complexity of genetic determinants of prion disease susceptibility. The cleavage of PrP^C may be a suitable therapeutic target in prion disease.

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PS.15: The role of host factors in prion strain interference

Katie A. Langenfeld and Jason C. Bartz

Creighton University; Omaha, NE USA

Background. Prion diseases are infectious neurodegenerative disorders that affect humans and other mammals and are inevitably fatal. The infectious agent in prion disease (PrP^{Sc}) is an abnormal isoform of an endogenous host protein (PrP^C). Prion conversion involves a conformational change of PrP^C into PrP^{Sc}, and this process may involve host polyanions such as ribonucleic acid (RNA). Prions have different strains that can interfere with one another and influence prion adaptation; however, the mechanism of this process is not well understood. During strain interference, a long incubation period (blocking) strain can interfere with the emergence of a short incubation period (superinfecting) strain. We hypothesize that RNA molecules are sequestered by blocking strains, preventing their use in conversion by superinfecting strains. Understanding the mechanism of prion strain interference would offer insight into the biology of prion transmission and adaptation.

Materials and Methods. To demonstrate the need for RNA in prion conversion, uninfected hamster brain homogenate was treated with RNase and then used as substrate in protein misfolding cyclic amplification (PMCA), an in vitro prion conversion assay. RNA was then added to the RNase treated homogenate to reconstitute the PMCA substrate. To examine the role of RNA in strain interference, RNA was added in excess to an in vitro strain interference model utilizing PMCA and two strains of hamster adapted transmissible mink encephalopathy (TME): drowsy (DY) TME (the blocking strain) and hyper (HY) TME (the superinfecting strain).

Results. The use of RNase treated PMCA substrate resulted in inhibited prion conversion, and the addition of RNA to RNase treated PMCA substrate rescued prion conversion. If RNA is the factor that HY and DY TME compete for, we predict that addition of RNA would enable the superinfecting strain (HY TME) to overcome the interference effect due to the availability of free RNA not sequestered by the blocking strain (DY TME). However, addition of RNA to the in vitro strain interference model did not result in the rapid emergence of HY TME indicating that RNA is not the factor that HY and DY TME compete for.

Conclusion. Based upon these results, RNA is not involved in prion strain interference. It is possible that the blocking strain (DY TME) does not sequester RNA but rather sequesters PrP^C or another host factor, leading to the inhibited emergence of the superinfecting strain (HY TME).

PS.16: Transmembrane anchoring of the prion protein in a Prnp knockout cell line: The effects of plasma membrane location on propagation of misfolded PrP

Karen E. Marshall

Rocky Mountain Laboratories; Hamilton, MT USA

Prion diseases are associated with misfolded aggregates of the prion protein (PrP), which are suspected as the primary pathological component and infectious agent. In its natively folded form PrP is anchored to the extracellular face of the plasma membrane by a glycophosphatidylinositol (GPI) moiety that inserts into, but does not traverse, the cell membrane. The GPI anchor directs PrP to distinct regions of the membrane termed lipid rafts that are rich in cholesterol and sphingomyelin. It has been proposed

that targeting to raft microdomains is important for formation of misfolded PrP and neurotoxic signaling.

To study how GPI anchoring and plasma membrane location affects PrP misfolding and other aspects of prion biology we have created cloned cell lines expressing transmembrane (TM) and wild-type (WT, i.e., GPI) anchored forms of the mouse prion protein (MoPrP). Replacing the GPI anchor with a transmembrane (TM) domain from a non-raft protein directs PrP to a different, non-lipid raft compartment. A fluorescent fusion protein of the TM-MoPrP construct was created to visualize any conversion and aggregation of PrP and associated cellular trafficking. The WT and TM-MoPrP constructs were stably transduced into NpL2 cells, a $Prnp^{-/-}$ mouse neuronal cell line and cloned to derive cell lines with good overall and, in particular, cell surface MoPrP expression. Only cells expressing WT MoPrP appeared to successfully convert following infection with either 22L or RML scrapie strains. However, both WT and TM-MoPrP cell lysates were able to seed conversion of recombinant mouse PrP in a cell-free conversion reaction, indicating that TM-MoPrP is converting into an alternative isoform not detectable using standard proteinase K (PK) conditions but observed with lower concentrations of PK. Thus we describe here a novel cell culture model that can be used to investigate and measure cell-derived infectivity. Furthermore, these results indicate that transmembrane anchored forms of mouse PrP are capable of converting into a non-native isoform with different characteristics to GPI anchored PrP, possibly as a result of its location within the plasma membrane.

PS.17: Deuterium exchange identifies dynamic protein folding events during infectious prion formation

Michael B. Miller[1,2] Daphne Wang,[3] Fei Wang,[4] Tong Liu,[3] Geoff P. Noble,[2] Jiyan Ma,[4] Virgil L. Woods,[3] Sheng Li[3] and Surachai Supattapone[2]

[1]Dartmouth Medical School; Hanover, NH USA; [2]Geisel School of Medicine at Dartmouth; Hanover, NH USA; [3]University of California at San Diego; La Jolla, CA USA; [4]Ohio State University; Columbus, OH USA

Creutzfeldt-Jakob disease, bovine spongiform encephalopathy, and chronic wasting disease are transmitted by infectious prions, which contain PrP^{Sc}, a protease-resistant form of the native cellular prion protein, PrP^{C}. Neither the precise composition of prions nor the atomic structure of PrP^{Sc} is known, though the structure of PrP^{C} has been determined. Non-protein cofactors have been found to be essential to the conversion of PrP^{C} into PrP^{Sc}, but the events underlying this conformational change are not clear. When mixed with phospholipid and polyanion cofactor molecules, PrP^{C} adopts an insoluble but non-infectious intermediate form, from which infectious prions can be generated by protein misfolding cyclic amplification (PMCA). Using deuterium exchange mass spectroscopy (DXMS) to assess the regions of protein exposed to solvent water molecules, we monitored structural changes occurring in the in vitro conversion process of PrP^{C} through the insoluble intermediate form to PrP^{Sc}. These results suggest that incubation with cofactor molecules causes an initial conformational change, which appears to then permit subsequent folding events that generate PrP^{Sc}. In addition to clarifying molecular events during prion protein conversion, these findings identify potential sites for molecularly targeted therapies to interrupt fatal propagation if infectious prions.

PS.18: Using drosophila to model the biology of the β2-α2 loop

Geoffrey Noble,[1] Patrick Dolph[2] and Surachai Supattapone[1]

[1]Geisel School of Medicine at Dartmouth; Hanover, NH USA; [2]Dartmouth College; Hanover, NH USA

In recent years, the β2-α2 loop has emerged as a region of the prion protein (PrP) in which amino acid substitutions can profoundly influence PrP misfolding. Studies in transgenic mice have shown that mutations in the loop can cause spontaneous prion disease or, conversely, can provide protection against prion inoculation.[1,2] Given the importance of this region in controlling the ability of PrP to misfold, a model system in which multiple genetic hypotheses can be tested rapidly in vivo is desirable.

Our lab has previously used Drosophila to model Gerstmann-Sträussler-Scheinker (GSS) syndrome, an inherited prion disease.[3] We have recently enhanced the robustness of our Drosophila PrP expression system by utilizing phiC31 integrase for site-specific transgene insertion, as well as the TARGET system for inducible protein expression, allowing for rapid and reproducible transgene expression in vivo.[4,5] With these genetic tools in hand, we are interested in using Drosophila as a model to recapitulate the biology of the β2-α2 loop. These studies may provide insight into the mechanisms by which the primary sequence of the β2-α2 loop controls PrP misfolding.

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PS.19: Discovery of peptides from bovine brain which accelerate structural conversions of the recombinant bovine prion protein

Kiyoshi Nokihara

HiPep Laboratoris; Kyoto, Japan

Proteins designated as molecular chaperones are known to assist structural conversion or folding. In contrast to the known effects of β-sheet breaker peptides,[1] we hypothesize that certain peptides may induce or enhance the conformational change of proteins as reverse phenomenon. Recently we have developed a structural conversion assay system with recombinant bovine prion protein (rbPrP)[2] to investigate the validity of the above hypothesis. We have shown that the co-existence of certain designed peptides influenced the kinetic rate of aggregation and the lag-time of fibril formation of rbPrP. The peptide library has been constructed from enzymatic digestion followed by chromatography of natural bovine brain obtained from a local slaughter house. Using the above conversion assay method, we have screened peptides responsible for structural conversion. Positive components were characterized by mass spectrometry and their sequences were determined. De novo generated peptide sequences were elucidated by using the MS BLAST algorithm. These peptides were then chemically synthesized to confirm their effects on both the lag time and rate of structural conversion of rbPrP. The peptide exhibited significant effects has been discovered which originates synaptophysin.[3] PrP^C is highly expressed at synapses,[4] although the structural conversion by the peptides derived from synaptophysin has not been described. The interaction between synaptophysin with PrP^C plays a role in synaptic function.[5] These findings support our present results. The structural elucidation of components in active mixtures can be directly analyzed using high resolution mass spectrometer to give multiple precursor ions in MALDI-TOF-MS which can be further analyzed by MS/MS to reveal the sequence of each component. All those peptides can be chemically synthesized to determine real active components. Such an approach allows minimization of the time required to discovery of active compounds. A dose-dependency was established using the synthetic peptide, because unknown amounts of target were assayed at the stage of screening of the natural library. We believe that more peptides responsible for structural conversion and peptides exhibiting opposite effects may exist in the brain to maintain homeostasis. Discovery of the peptide which accelerates structural conversions of rbPrP is described.

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PS.20: Effect of fibrillation on copper binding to prion protein

Xu Qi and Michele McGuirl

University of Montana; Missoula, MT USA

Conversion of the prion protein from its α-rich form (PrP^{Sen}) to the β-rich scrapie form (PrPRes) constitutes the key event of the etiology of prion diseases. Fundamental questions remain concerning the physiological roles of PrP^{Sen}. There is a wealth of evidence links PrP^{Sen} function to its ability to bind Cu(II). In vitro experiments have revealed that PrP^{Sen} binds four Cu(II) in its octarepeat region and another at the fifth binding site extrinsic to the octarepeat region. Detailed structural information on copper binding to these sites in PrP^{Sen} has been demonstrated using electron paramagnetic resonance (EPR) spectroscopy and other techniques. However, much less attention has been devoted to understanding copper binding in PrPRes, which is more pathologically relevant. To address this question, we have investigated the effect of fibrillation on copper binding in PrP. Our data show that PrPRes preserves the ability to bind Cu(II). Low temperature X-band EPR spectra indicate that in PrPRes both octarepeat region and the 5th site bind Cu(II) with coordination modes similar to their monomeric counterparts, with 5–6 Cu(II) per protein under saturation conditions. When Cu(II) binding to PrP^{Sen} is measured at room temperature by isothermal titration calorimetry (ITC), multiple Cu(II) ions bind and the protein begins to aggregate, In stark contrast, ITC measurements of Cu(II) binding to PrPRes yield a stoichiometry of only 1 Cu(II) ion. To better understand the apparently conflicting data, we are now studying the characteristics of Cu(II) binding to PrPRes and PrP^{Sen} by room temperature EPR.

PS.21: Introducing a rigid loop structure from deer into mouse prion protein increases its propensity for misfolding in vitro

Leah M. Kyle,[1] Theodore R. John,[1] Randolph V. Lewis[2] and Hermann M. Schätzl[3]

[1]University of Wyoming; Laramie, WY USA; [2]Utah State University; North Logan, UT USA; [3]University of Calgary; Calgary, AB Canada

Prion diseases are fatal neurodegenerative disorders characterized by misfolding of the cellular prion protein (PrP^c) into the disease-associated isoform PrP^{Sc} that has increased β-sheet content and partial resistance to proteolytic digestion. Prion diseases from different mammalian species have varying propensities for

transmission upon exposure of an uninfected host to the infectious agent. Chronic Wasting Disease (CWD) is a highly transmissible prion disease that affects free ranging and farmed populations of cervids including deer, elk, and moose as well as other mammals in experimental settings. The mechanism allowing CWD to maintain comparatively high transmission rates has not been determined. Previous work has identified a unique structural feature in cervid PrP, a rigid loop between β-sheet 2 and α-helix 2 on the surface of cervid PrP. This study was designed to test the hypothesis that the rigid loop has a direct influence on the misfolding process. The rigid loop was introduced into murine PrP as the result of two amino acid substitutions: S170N and N174T. Wild-type and rigid loop murine PrP were expressed in *E. coli* and purified. Misfolding propensity was compared for the two proteins using biochemical techniques and cell free misfolding and conversion systems. Current findings indicate that murine PrP with a rigid loop misfolds in cell free systems with greater propensity than wild type murine PrP. In a lipid-based conversion assay, rigid loop PrP converted to a PK resistant, aggregated isoform at lower concentrations than wild-type PrP. Using both proteins as substrates in real time quaking-induced conversion, rigid loop PrP adopted a misfolded isoform more readily than wild type PrP. These findings may help explain the high transmission rates observed for CWD within cervids.

PS.22: The PrP-like ZIP5 ectodomain co-localizes with the prion protein and exists as a dimer

Cosmin L. Pocanschi,[1] Sepehr Ehsani,[1] Mohadeseh Mehrabian,[1] William Reginold,[1] Hansen Wang,[1] Holger Wille,[2] Adelinda Yee,[3] Cheryl H. Arrowsmith,[3] Zoltan Bozoky,[3] Julie D. Forman-Kay,[3] Lewis E. Kay,[3] James M. Rini[3] and Gerold Schmitt-Ulms[1]

[1]Tanz Centre for Research in Neurodegenerative Diseases; Toronto, ON Canada; [2]University of Alberta; Edmonton, AB Canada; [3]University of Toronto; Toronto, ON Canada

The cellular prion protein (PrP^C) was recently observed to co-purify with members of the LIV-1 subfamily of ZIP zinc transporters (LZTs), a finding which precipitated the surprising discovery that the prion gene family descended from an ancestral LZT gene. Here, we compare the cellular distribution and biophysical characteristics of LZTs and their PrP-like ectodomains. When expressed in neuroblastoma cells the ZIP5 member of the LZT subfamily was observed to be directed to largely identical molecular environments as PrP^C, and both proteins were seen to be endocytosed through vesicles decorated with the Rab5 marker protein. When recombinantly expressed, the PrP-like domain of ZIP5 could be obtained with yields and at levels of purity sufficient for structural analyses but tended to aggregate, thereby precluding attempts to study its native structure by spectroscopic methods. These obstacles were overcome by moving to a mammalian expression system. Once a post-translationally glycosylated ZIP5 ectodomain was secreted into the medium, the enzymatic removal of its N-glycans was well-tolerated by this expression product. The method also allowed the expression and purification of stable preparations of Tr_PrP-1, thereby overcoming a key hindrance to high-resolution structural work for fish PrP. The subsequent biophysical characterization of a homogeneous preparation of the PrP-like ectodomain of ZIP5 documented that this domain acquires a dimeric, largely globular fold with similar α-helical content as PrP^C.

PS.23: Amplification of infectious prion strains in vitro

Ronald A. Shikiya and Jason C. Bartz

Creighton University; Omaha, NE USA

Prions are infectious agents that cause the transmissible and familial forms of spongiform encephalopathy in animals and humans. The infectious prion agent is mainly, if not entirely, comprised of a protein (PrP^{Sc}) and is able to propagate by inducing misfolding on the host's constituent prion protein (PrP^C) and produce neurodegeneration.

Several prions strains that breed true upon experimental passage have been identified. Prion strains are defined by their distinctive set of phenotypic properties of disease such as incubation period, neuropathology, agent distribution and biochemical characteristics of PrP^{Sc} that can include, conformational stability, and fibrillar structure.

Protein Misfolding Cyclic Amplification (PMCA) is a powerful technique that effectively converts PrP^C to PrP^{Sc} in vitro. PMCA uses sonication and incubation cycles to accelerate the propagation of a PrP^{Sc} seed within a PrP^C rich environment. One of the main advantages of PMCA is that great quantities of PrP^{Sc} can be produced in a short period of time.

In this study we use serial PMCA to amplify two well-characterized hamster adapted prion strains, HY TME and DY TME. Several PMCA rounds were conducted to dilute out of the solution the original brain-derived PrP^{Sc} seed leaving only the in vitro generated PrP^{Sc}.

Animals inoculated with the PMCA-generated PrP^{Sc} developed similar clinical sings and neuropathology when these traits were compared with their brain-derived counterparts. In addition, their biochemical characteristics such conformation stability, amplification coefficient and electrophoretic mobility correspond to those of their brain-derived PrP^{Sc}. Positive controls demonstrate that sonication and incubation cycles does not affect the incubation period of HY TME suggesting that the PMCA itself does not affect infectivity. An endpoint dilution analysis shows that the PMCA-generated HY TME has a titer similar to that of its brain derived counterpart. This indicates that PMCA is able to amplify prion strains and the amplified material is highly infectious.

PS.24: Anchored and anchorless prions show major differences in their biochemical features and neuroinvasion ability

Christina Sigurdson,[1] Cyrus Bett,[1] Tim Kurt,[1] Melanie Lucero,[1] Margarita Trejo,[1] Annemieke Rozemuller,[3] Qingzhong Kong,[2] K. Peter R. Nilsson,[4] Eliezer Masliah[1] and Michael B. Oldstone[5]

[1]University of California, San Diego; La Jolla, CA USA; [2]Case Western University; Cleveland, OH USA; [3]University Medical Center Utrecht; Utrecht, The Netherlands; [4]Linkoping University; Linkoping, Sweden; [5]The Scripps Research Institute; La Jolla, CA USA

Infectious prions show an extraordinary range of structures, from amorphous aggregates to fibrils, and cause diverse clinical signs. How the conformation of a prion dictates the disease phenotype remains unclear. Mice expressing GPI-anchorless or GPI-anchored prion protein exposed to the same infectious prion have been previously found to develop fibrillar or nonfibrillar aggregates, respectively, and show a striking divergence in the disease pathogenesis. To better understand how a prion's physical properties govern pathogenesis, we passaged infectious anchorless prions in mice expressing anchorless cellular prion protein and biochemically characterized the resulting prions. We found that serial passage of infectious anchorless prions led to a significant decrease in the incubation period and altered biochemical properties, consistent with a transmission barrier effect. After an intraperitoneal exposure, anchorless prions were only weakly neuroinvasive, as prion plaques rarely occurred in the brain yet were abundant in extracerebral sites such as spleen, heart, and adipose tissue. Compared with the corresponding anchored prions, anchorless prions consistently showed very high stability in chaotropes or when heated in SDS, and were highly resistant to enzyme digestion. Interestingly, anchorless prions from a human patient were also highly stable in chaotropes, consistent with the results in mice. Thus our results indicate that nonfibrillar GPI-anchored prions can convert into fibrillar GPI-anchorless prions that are highly stable, enzyme resistant, and no longer able to efficiently invade the central nervous system.

PS.25: Distinguishing between PrP^{C} and PrP^{Sc} using small molecule reagents

Christopher J. Silva, Melissa L. Erickson, Irina Dynin and J. M. Carter

USDA; Albany, CA USA

Background and Introduction. The structural difference between PrP^{Sc} and PrP^{C} is entirely conformational: they are isoforms. Both isoforms possess identical covalent structures and identical post-translational modifications. This means that the same amino acid can react differently with the same chemical reagent, depending upon which of the isoforms is reacted. The site of covalent modification can be identified by mass spectrometry or by western blot, if the epitope of the primary antibody contains an amino acid that can be covalently modified by a selected reagent.

Materials and Methods. A set of small molecules reagents was synthesized. These reagents preferentially react with the ε-amino group of lysine. They were reacted with PrP^{Sc}, PrP^{C}, and recombinant PrP. The reaction mixtures were analyzed by mass spectrometry and by western blot to determine the relative reactivity of the various lysines. Four of the antibodies we used recognize an epitope that is encrypted in the PrP^{Sc} isoform, but exposed in the PrP^{C} isoform.

Results and Conclusion. Because these reagents block the recognition of PrP^{C}, western blot analysis of these reactions permits the detection of prion infected brain extracts without the need for proteinase K digestion. This is important, because although proteinase K-resistant PrP is diagnostic for disease, much of infectious PrP is proteinase K-sensitive. In addition these reagents can be used, with an appropriate antibody, to determine which amino acids of PrP^{Sc} are exposed on the surface and which are encrypted, thus providing useful structural information. This approach was used to distinguish among strains of hamster-adapted scrapie without the use of proteinase K. The mass spectrometry-based analysis was used to quantitate these differences and analyze the relative reactivity of the various lysines present in hamster PrP.

PS.26: A comparison of the structure of the PK-sensitive and PK-resistant forms of PrP^{Sc}

Christopher J. Silva,[1] Gustavo Sajnani,[2] Adriana Ramos,[2] Miguel A. Pastrana,[2] Bruce C. Onisko,[3] Melissa L. Erickson,[1] Elizabeth M. Antaki,[1] Irina Dynin,[1] Ester Vázquez-Fernández,[2] Christina J. Sigurdson,[4] J. M. Carter[1] and Jesús R. Requena[2]

[1]USDA; Albany, CA USA; [2]Department of Medicine; School of Medicine; University of Santiago de Compostela; Santiago de Compostela, Spain; [3]OniPro Biosciences; Kensington, CA USA; [4]Department of Pathology; University of California, San Diego; La Jolla, CA USA

Background and Introduction. One of the distinctive phenotypes of the infectious isoform of PrP (PrP^{Sc}) is its resistance to proteinase K (PK) digestion. The diagnosis of prion diseases is based on this phenotypic observation. More recently, researchers determined that there is a sizeable fraction of PrP^{Sc} that is sensitive to PK hydrolysis ($sPrP^{Sc}$). We wished to determine if there was a phenotypic or biochemical difference between PrP^{Sc} and the sub-fractions of PK-resistant PrP^{Sc} ($rPrP^{Sc}$) and $sPrP^{Sc}$.

Materials and Methods. We used our previously reported method of isolating PrP^{Sc}, $sPrP^{Sc}$ and $rPrP^{Sc}$. These three fractions were bioassayed to compare their relative infectivity using the 263K strain of hamster-adapted scrapie. In addition we performed an immunohistochemical analysis of the three forms to determine whether they displayed the same pathology. The structural characteristics of the three forms were analyzed by mass spectrometry (MS) of the partial PK digest of these fractions. In addition we used partial PK digestion/MS to analyze

the structures, $sPrP^{Sc}$ and $rPrP^{Sc}$, of the drowsy (Dy) strain of hamster-adapted scrapie.

Results and Conclusion. The $sPrP^{Sc}$ and $rPrP^{Sc}$ fractions from the 263K strain have comparable degrees of infectivity and although they contain different sized multimers, these multimers share similar structural properties. Histopathological and immunohistochemical analyses of brains from animals inoculated (ic) with these fractions all showed an identical pathology. Furthermore, the PK-sensitive fractions of two hamster strains, 263K and Dy, showed strain-dependent differences in the ratios of the $sPrP^{Sc}$ to the $rPrP^{Sc}$ forms of PrP^{Sc}. Thus, although the $sPrP^{Sc}$ and $rPrP^{Sc}$ fractions have different resistance to PK-digestion, have previously been shown to sediment differently, and have a different distribution of multimers, they share a common structure and phenotype.

PS.27: The region between helix 1 and 2 is critically important for efficient dominant-negative inhibition (DNI) by conversion-incompetent PrP

Yuzuru Taguchi and Hermann M. Schätzl

University of Wyoming; Laramie, WY USA

A profound conformational change of PrP^{C} underlies formation of PrP^{Sc} and prion propagation involves conversion of PrP^{c} substrate by direct interaction with PrP^{Sc} template. Identifying the interfaces and modalities of intermolecular interactions of PrPs will highly advance our understanding of prion propagation in particular and of prion-like mechanisms in general. To identify the region critical for intermolecular interactions of PrP, we exploited here dominant-negative inhibition (DNI) effects of conversion-incompetent, internally-deleted PrP (ΔPrP) on co-expressed conversion-competent PrP. We created a series of ΔPrPs with different lengths of deletions in the region between first and second α-helix (H1~H2) which was recently postulated to be of importance in prion species barrier and PrP fibril formation. As previously reported, ΔPrPs uniformly exhibited aberrant properties including detergent insolubility, limited protease digestion resistance, high-mannose type N-linked glycans, and intracellular localization. Although formerly controversial, we demonstrate here that ΔPrPs have a GPI anchor attached. Surprisingly, despite very similar biochemical and cell-biological properties, DNI efficiencies of ΔPrPs varied significantly, dependant on location and inversely correlated with the size of deletion. This data demonstrates that H1~H2 and its relative positioning to the region C-terminal to it are critical determinant of efficient DNI. It also implies that this region is possibly involved in PrP-PrP interaction and conversion of PrP^{C} into PrP^{Sc}. Further investigations with ΔPrPs might identify how PrP^{C} and PrP^{Sc} interact and that might also provide a new therapeutic target. Besides, to reconcile the paradox of how an intracellular PrP can exert DNI, we demonstrate that ΔPrPs are subject to both proteasomal and lysosomal/autophagic degradation pathways. Using autophagy pathways ΔPrPs obtain access to the locale of prion conversion and PrP^{Sc} recycling and can exert DNI there. This shows that the intracellular trafficking of PrPs is more complex than previously anticipated.

PS.28: Structural consequences of native post-translational modifications of the prion protein: A mechanism for toxicity

Lara M. Taubner

University of Montana; Missoula, MT USA

Prion diseases are "protein-only" fatal neurodegenerative diseases associated with the massive structural transformation of the normally soluble, GPI-anchored prion protein monomer (PrP^{sen} for protease sensitive) into an aggregated, insoluble protein high in β sheet content (PrP^{res} for protease resistant). There is considerable debate regarding the actual cause of neuronal death; current thinking is that PrP^{res} may propagate the disease but not be the actual toxic agent. Recent in vivo studies suggest that uncontrolled cation flux is in part the cause of cell death. We hypothesize that the observed ion current is due to direct interactions of PrP^{sen} with the plasma membrane rather than via the modulation of endogenous ion channels. To investigate this problem, we have characterized the wild type human prion protein and a mutant that lacks the hydrophobic domain (or core region/ΔCR) that has been shown to induce cation flux and is lethal to Tg mice. In contrast to previous structural studies of PrP^{sen} using protein that has been refolded from inclusion bodies produced in *E. coli*, we have chosen to study PrP^{sen} expressed by the eukaryotic host Pichia pastoris. In this host, both wild type and deletion mutant ΔCR PrP^{sen} are associated with the cytoplasmic membrane fraction via the GPI anchor. We observe three distinct glycoforms in a pattern similar to that of mammalian PrP^{sen}. Circular Dichroism spectra of purified wild type and mutant PrP^{sen} in detergent micelles show that both proteins exhibit at least 15% more helical secondary structure than PrP^{sen} from *E.coli* even when equivalent micellar concentrations of detergent are used. These results suggest that membrane tethering and/or posttranslational modifications of PrP^{sen} stabilize structural elements of the protein that may be disordered in PrP^{sen} refolded from *E.coli*. Therefore, PrP^{sen} produced in yeast may be a better model for studying both the normal function and the postulated cation flux mechanism of PrP.

PS.29: Dual localization of the prion protein family members: the highly conserved, N-terminal (RXXX)8 motif of mouse shadoo mediates nuclear accumulation

Eszter Tóth,[1,2] Péter I. Kulcsár,[1] Elfrieda Fodor,[1] Ferhan Ayaydin,[1] Lajos Kalmár,[3] Adrienn É. Borsy,[4] Lajos László[2] and Ervin Welker[1,4]

[1]HAS Biological Research Center; Szeged, Hungary; [2]ELTE Faculty of Science; Department of Anatomy; Cell and Developmental Biology; Budapest, Hungary; [3]HAS Research Centre for Natural Sciences; Institute of Enzymology; Budapest, Hungary; [4]HAS Research Centre for Natural Sciences; Institute of Molecular Pharmacology; Budapest, Hungary

The prion protein (PrP)—known for its central role in transmissible spongiform encephalopathies—has been reported to possess two nuclear localization signals and localize in the nuclei of certain cells in various forms. Although these data are superficially contradictory, it is apparent that nuclear forms of the prion protein can be found in cells in either the healthy or the diseased state. There are growing numbers of evidences about the appearances of various intracellular forms of the prion protein, which might play a role in prion diseases. In this respect, it is intriguing that another prion protein superfamily member—the Shadoo (Sho)—is also found in the nucleocytoplasm of some neurons.[1]

Here we report the accumulation of Shadoo in the nucleus of several neural and non-neural cell lines as visualized by using an YFP-Sho construct. This nuclear localization is mediated by the (25–61) fragment of mouse Sho encompassing an (RXXX)8 motif. Bioinformatic analysis shows that the (RXXX)n motif (n = 7–8) is a highly conserved and characteristic part of mammalian Shadoo proteins. Experiments to assess if Shadoo enters the nucleus by facilitated transport gave no decisive results; however, these experiments revealed that Shadoo's interactions in the nucleolus and in the rest of the nucleus are markedly different.[2] The most pivotal questions are tracing the journey of various forms of Shadoo from translation to the nucleus and discerning the potential nuclear function and binding partner(s) of PrP and Shadoo.

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PS.30: Targeted interactomics to characterize anchorless 23–230 form of PrP^C

Saima Zafar[1] Abdul R. Asif,[2] Sanja Ramljak,[1] Waqas Tahir,[3] Matthias Schmitz[3] and Inga Zerr[3]

[1]Institute for Clinical Research and Development (ikfe); Mainz; Germany; Mainz, Germany; [2]Department of Clinical Chemistry; Goettingen, Germany; [3]Clinical Dementia Center; Department of Neurology and Psychiatry; Goettingen, Germany

Keywords: anchorless PrP^C, proteomics, pyruvate kinase isozymes M1/M2, prion protein, interactomics.

In prion and other neurodegenerative diseases, the accumulation of conformationally altered cellular proteins is a common feature. Recent reports demonstrated that the absence of glycosylphosphatidylinositol (GPI) lipid anchor could lead to a protease-resistant conformation that resembles scrapie associated isoform of prion protein (PrP^{Sc}). Therefore, the physio-pathological function of anchorless 23–230 PrP^C (Δ23–230 PrP^C) has come into focus of attention. In this study we aimed to uncover the physiopathological function of the anchorless PrP^C form by identifying its interacting partner proteins. The anchorless Δ23–230 PrP^C along with its interacting proteins was affinity purified using STrEP-Tactin-chromatography, in-gel digested, and identified by Q-TOF MS/MS analysis in prion protein-deficient murine hippocampus (HpL3–4) neuronal cell line. Twenty four proteins appeared to interact with anchorless Δ23–230 PrP^C in HpL3–4 cells. Out of 20 four proteins two novel proteins (1) Ras-related protein Rab-1a (Rab-1a) and (2) Pyruvate kinase isozymes M1/M2 (PKM2) exhibited a potential interaction with the anchorless Δ23–230 form of PrP^C. Both, reverse co-immunoprecipitation and confocal laser scanning microscopic analysis confirmed an interaction of Rab-1a and PKM2 with the anchorless Δ23–230 form of PrP^C. Furthermore, we functionally characterize PKM2 associated expressional regulation during apoptotic stress condition. In conclusion our data highlights that the misbalance of anchorless Δ23–230 form of PrP^C in association with the expressional regulation of interacting proteins could render cells more prone to cellular insults and may ultimately are linked to prion neurotoxicity.

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PS.31: Unglycosylated recombinant prion protein inhibits homologous prion propagation in vitro: A glycoform barrier?

Jue Yuan,[2] Yi-An Zhan,[2] Romany Abskharon,[4] Xiangzhu Xiao,[2] Manuel Camacho Martinez,[2] Geoff Kneale,[5] Jacqueline Mikol,[6] Sylvain Lehmann,[7] Joaquín Castilla,[8] Jan Steyaert,[4] Qingzhong Kong,[2] Robert B. Petersen,[2] Alexandre Wohlkonig[4] and Wen-Quan Zou[1,2]

[1]Case Western Reserve University; Cleveland, OH USA; [2]CWRU; Cleveland, OH USA; [3]VIB; Department of Structural Biology; Structural Biology Brussels; Vrije Universiteit Brussels; Blussel, Belgium; [4]Vrije Universiteit Brussels; Brussels, Belgium; [5]University of Portsmouth; Portsmouth, UK; [6]Service d'Anatomie et Cytologie Pathologiques; Paris, France; [7]IRB - Hôpital ST ELOI; Montpellier, France; [8] CIC bioGUNE and IKERBASQUE; Basque Foundation for Science; Bizkaia, Spain

Prion diseases are associated with the conformational transition of the cellular prion protein (PrP^{C}) into a pathological scrapie isoform (PrP^{Sc}) in the brain. Although the molecular mechanism underlying the conversion remains unsolved, it has been well-documented that both in vivo and in vitro the conversion of PrP^{C} into PrP^{Sc} is significantly influenced by differences in amino acid sequence between the two species, indicating the presence of an amino acid barrier. Our recent work also demonstrated a glycoform-selective prion formation in sporadic and familial forms of prion disease, suggesting the presence of a glycoform barrier. Using protein misfolding cyclic amplification (PMCA), a cell-free assay that has been widely used to mimic in vivo prion propagation, we now report that the homologous unglycosylated recombinant full-length human PrP is able to completely inhibit human prion propagation and the half maximal effective concentration (EC_{50}) is approximately 60 nM while EC_{50} of recombinant full-length mouse PrP for mouse prion 139A is about 120 nM. Furthermore, unglycosylated human PrP also inhibits mouse prion propagation in a scrapie-infected mouse cell line (ScN2a). Notably, the unglycosylated recombinant PrP binds to PrP^{Sc}, but not PrP^{C}, suggesting that the inhibitory effect is attributable to blocking the interaction of brain PrP^{C} and PrP^{Sc} by the unglycosylated PrP. These results favor the hypothesis that the different ratio of PrP^{C} glycoforms by increasing unglycosylated PrP^{C} modulates the efficiency of prion formation. Our findings may suggest new avenues for developing therapeutic targets for prion diseases. (Supported by the National Institutes of Health (NIH) NS062787, NIH AG-14359, the CJD Foundation, and CDC Contract UR8/CCU515004.)

PS.32: Role of quiescin-sulfhydryl oxidase in prion formation

Yi-An Zhan,[2] Romany Abskharon,[3] Jue Yuan,[2] Manuel Camacho Martinez,[2] Xiangzhu Xiao,[2] Jacqueline Mikol,[4] Sylvain Lehmann,[5] Jan Steyaert,[3] Qingzhong Kong,[2] Robert B. Petersen,[2] Alexandre Wohlkonig[3] and Wen-Quan Zou[1,2]

[1]Case Western Reserve University; Cleveland, OH USA; [2]CWRU; Cleveland, OH USA; [3]Vrije Universiteit Brussels; Brussels, Belgium; [4]Service d'Anatomie et Cytologie Pathologiques; Paris, France; [5]IRB—Hôpital ST ELOI; Montpellier, France

Prions are infectious pathogens that cause transmissible diseases in animals and humans. The scrapie form (PrP^{Sc}) of the cellular prion protein (PrP^{C}) is the only known component of the prion. Several lines of evidence have suggested that the formation and molecular features of PrP^{Sc} are associated with as yet unknown abnormal processes that unfold and refold PrP^{C}. Quiescin-sulfhydryl oxidase (QSOX) is believed to play a role in protein folding by introducing disulfides into unfolded, reduced proteins. Here we demonstrate that QSOX inhibits human prion propagation in cell-free protein misfolding cyclic amplification and that it also inhibits murine prion propagation in scrapie-infected neuroblastoma cells (ScN2a). Moreover, QSOX preferentially binds to PrP^{Sc} from prion-infected human or animal brains, but not PrP^{C} from uninfected brain. Surface plasmon resonance (SPR) of recombinant mouse PrP (rMoPrP) demonstrates that the affinity of QSOX for PrP monomers is significantly lower than that for PrP octamers (312 nM vs 1.7 nM). QSOX exhibits much lower affinity for N-terminally truncated PrP (PrP89–230) than for full-length PrP (PrP23–231) (312 nM vs 2 nM). The affinity of QSOX is also much lower for N-terminally truncated human PrP90–231 than for an N-terminal human PrP fragment (PrP23–145) (771 nM vs 3.6 nM), suggesting that N-terminal region of PrP is largely responsible for the interaction of PrP with QSOX. Our study indicates that QSOX may play a role in prion formation, potentially opening new avenues for developing therapeutics to treat prion diseases. (Supported by the National Institutes of Health (NIH) NS062787, NIH AG-14359, the CJD Foundation, and CDC Contract UR8/CCU515004.)

PS.33: Structural studies on the folded domain of the prion protein bound to the Fab fragment of the antibody POM1

Pravas K. Baral,[1] Tiziana Sonati,[2] Barbara Wieland,[3, 1] Mridula Swayampakula,[1, 3] Magdalini Polymenidou,[4] Nat N. Kav,[3] Adriano Aguzzi,[2] and Michael N. James,[1]

[1]Department of Biochemistry; University of Alberta; Edmonton; Edmonton, AB Canada; [2]Department of Pathology; Institute of Neuropathology; University Hospital; Zurich, Switzerland; [3]Department of Agricultural; Food and Nutritional Science; University of Alberta; Edmonton AB, Canada; [4]Department of Cellular and Molecular Medicine; University of California at San Diego; San Diego, CA USA

Prion diseases are neurodegenerative diseases characterized by the conversion of the cellular prion protein PrP^{c} into a pathogenic isoform PrP^{Sc}. One of the therapeutic approaches for prion diseases is the passive immunization of anti-prion monoclonal antibodies as these molecules can stabilize the cellular prion protein in its native conformation and will arrest the progression of prion diseases. However rapid neurodegeneration in mice and in cerebellar organotypic culture slices is observed upon exposure of antibodies targeting the globular domain of the prion protein. The crystal structure of the Fab fragment of an anti-prion monoclonal antibody, POM1, in complex with the globular domain of human prion protein ($huPrP^{c}$) and mouse prion protein ($moPrP^{c}$) has been determined to a resolution of 2.4Å and 1.9Å, respectively. The prion epitope of POM1 is in close proximity to the epitope recognized by the purportedly therapeutic antibody fragment, ICSM18 Fab also with $huPrP^{c}$. POM1 Fab forms a 1:1 complex with the $huPrP^{c}$ and the measured K_d of 4.5 × 10–7 M reveals a moderately strong binding between them. Structural comparisons have been made among three prion-antibody complexes, POM1 Fab: $huPrP^{c}$, ICSM18 Fab: $huPrP^{c}$ and VRQ14 Fab: $ovPrP^{c}$. The prion epitopes recognized by ICSM18 Fab and VRQ14 Fab are adjacent to a prion glycosylation site, indicating possible steric hindrance and/or an altered binding mode with the glycosylated prion protein in vivo. However, both the glycosylation sites on $huPrP^{c}$ are located away from the POM1 Fab binding epitope, so the binding mode observed in this crystal structure and the binding affinity measured for this antibody will most likely be the same as with the native prion protein.

PS.34: Thermodynamic stability of amyloid fibrils and β-sheet oligomers: Perspective of molecular theory of solvation

Nikolay Blinov,[1,2] Wenjuan Huang,[1,2] David S. Wishart[1,2] and Andriy Kovalenko[1, 2]

[1]National Institute for Nanotechnology; Edmonton, AB Canada; [2]University of Alberta; Edmonton, AB Canada

Amyloid fibrils are a hallmark of many neurodegenerative diseases, including Alzheimer and Parkinson diseases, the mad cow disease and other transmissible spongiform encephalopathies.[1] The formation of amyloid fibrils is a particular example of self-assembly of macromolecules and as such is govern by general principles of self-assembly with the solvation effects playing a crucial role at all stages of the process.[5] As an alternative to the conventional solvation models, we have recently proposed to use the three dimensional molecular theory of solvation (3D-RISM-KH approach[3]) to describe the solvation effects at different levels of modeling [6.] Here, we apply the 3D-RISM-KH theory to assess thermodynamic stability of prion and Aβ oligomers and amyloid fibrils, to predict and characterize binding modes of prion proteins.[7-9]

We present the results on association thermodynamics of amyloid fibrils and β-sheet oligomers, as well as microscopic solvation structure of Alzheimer Aβ-peptides and prion proteins.[8,9] The point mutations reducing the system's charge and modification in the protonation state of ionizable residues of aggregates result in significant changes in electrostatic parts of the solvation and direct (gas-phase) contributions to the association free energy which are mostly mutually compensated. As a consequence, the overall thermodynamic stability of fibrils and oligomers, as well as pathways of aggregation are defined by a fine balance between solvation and gas-phase energetics, and are strongly affected by the change in the specific interactions (such as, for example, the disruption of salt bridges in proximity of mutation sites), through changes in energy and conformation space of the aggregates.[8,9] Both the solvation entropic and enthalpic effects are equally accounted for in the analysis. Additionally to the solvation thermodynamics, the miscroscopic solvation structure around the fibrils and oligomers is discussed. It is shown that amyloid fibrils can be regarded as water-filled nanotubes as suggested earlier. Additional to water channels predicted in previous molecular dynamics simulations, we describe possible formation of ion channels/locking structural ions and water molecules inside the fibril core.[10,11]

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PS.35: Unique misfolding mechanisms of human prion pathogenic mutants: Y218N and E196K

Chin Jung Cheng and Valerie Daggett

Department of Bioengineering; University of Washington; Seattle, WA USA

Prion diseases are caused by misfolding of the prion protein PrP. Pathogenic mutations such as Y218N and E196K are known to cause Gerstmann-Sträussler-Scheinker Syndrome and Creutzfeldt-Jakob disease, respectively. Here we describe molecular dynamics simulations of these mutant proteins to better characterize the detailed conformational effects of these sequence substitutions. Our results indicate that the mutations disrupt the wild-type native PrP structure and cause misfolding. Y218N lost hydrophobic packing around the X-loop and E196K abolished an important wild-type, native salt bridge. Despite the obvious differences at the mutation sites, we were able to observe common traits of misfolding for both mutants, but different pathways of triggering the misfolding events. Common traits of misfolding include: (1) HA detachment from the PrP core; (2) exposure of side chain F198; and (3) formation of a new strand at the flexible N-terminus. The effect of the E196K mutation was straightforward; the loss of the wild-type salt bridge E196-R156 destabilized the F198 hydrophobic pocket and the HA helix. The Y218N mutation propagated its effect by increasing the HB-HC interhelical angle, which eventually disrupted the packing of F198. Furthermore, a nonnative contact formed between E221 and the S132 on the S1-HA loop that offered a direct mechanism for disrupting the hydrophobic packing between S1-HA loop and HC. While there were common misfoldng features shared between Y218N and E196K, the differences in the orientation of HB and HC and the X-loop conformation might provide a structural basis for identifying different prion strains.

PS.36: Initial prion nucleation in the yeast-based assay

Yury O. Chernoff, Pavithra Chandramowlishwaran, Meng Sun and Zachery J. Deckner

Georgia Institute of Technology; Atlanta, GA USA

Self-perpetuating ordered cross-β protein aggregates (amyloids) are associated with a variety of mammalian and human disorders, including prion diseases and Alzheimer disease. Mechanism of the initial prion/amyloid nucleation remains unknown. Yeast prions, that control phenotypically detectable traits, are not homologous to known mammalian amyloid or prion proteins. Yeast system allows for simple phenotypic detection of initial prion nucleation. De novo formation of a yeast prion can be induced by transient overproduction of a prion-forming protein (or its prion domain) in the presence of other pre-existing prions. Apparently, pre-existing prions serve as heterologous nuclei for the generation of new prions by a different protein. We have shown that a fusion of the prion domain of the yeast prion protein to fragments derived from various mammalian prionogenic or amyloidogenic proteins (including PrP and Abeta) promotes de novo nucleation of a yeast prion in the absence of any pre-existing prions. Amyloid-like aggregation of mammalian proteins in yeast is also confirmed by biochemical techniques. Alterations of PrP or Abeta proteins abolishing their prionogenic or amyloidogenic properties in the mammalian or in vitro systems also eliminate prion nucleation in yeast. In contrast, truncated PrP derivatives associated with a heritable prion disease in humans exhibit significantly increased prion nucleation properties in yeast. The 42 amino acid derivative of Abeta (Abeta42) exhibits much higher prion nucleation efficiency, compared with the 40 amino acid derivative of Abeta (Abeta40). This agrees with the proposed roles of Abeta40 and Abeta42 in protein aggregation associated with Alzheimer disease. Notably, a spectrum of prion "strains" nucleated in yeast depends on the nucleating protein, indicating that mammalian amyloidogenic sequences play a crucial role in determining the structural parameters of resulting yeast prions. Our data establish a yeast-based experimental assay for studying initial prion nucleation and generation of strain-specific prion patterns. This assay can also be applied to identification of new prionogenic and amyloidogenic proteins of various origins.

PS.37: A novel mechanism for disrupting PrP^{Sc} accumulation in N2a22LSc cells

Valerie C. Cullen,[1] Ayodeji Asuni,[2] Rajaraman Krishnan,[1] Eva Asp,[1] Ming Prochitsky,[1] Kimberley Gannon,[1] and Richard Fisher,[1]

[1]NeuroPhage Pharmaceuticals; Cambridge, MA USA; [2]University of Southampton; Southampton, UK

Background. Misfolded protein aggregates play a central role in the pathobiology of neurodegenerative diseases, including prion diseases. Using biophysical and biochemical methods, we established that purified, formulated filamentous bacteriophage M13 (NPT002) directly and potently destabilizes and dissociates a broad class of amyloids, including yeast prion, as well as amyloid-β, tau, and α-synuclein. NPT002 also clears aggregate load in mouse models of Alzheimer and Parkinson Disease. Recently, we isolated the protein motif from NPT002 responsible for the amyloid interacting activity, which we call the generic amyloid interaction motif or GAIM. We constructed an IgGFc-GAIM fusion protein, and tested whether it can interfere with

formation of pathological prion conformers of PrP^{Sc} in in vitro models of prion propagation.

Materials and Methods. Amyloid fiber disaggregation was assessed using filter retardation assays. N2a22LSc cells were generated by infecting N2a cells with brain homogenate from mice infected with mouse-adapted 22L prion strain. Cells were treated for 24 h with increasing concentrations of IgGFc-GAIM, then lysed and analyzed by western blotting with and without Proteinase K (PK) digestion.

Results. Filter retardation assays show that NPT002 increases solubility of multiple aggregating proteins, including yeast prion protein, as well as Aβ, α-synuclein, and tau. Since increased detergent insolubility precedes acquisition of PK resistance by PrP^{Sc} or PrP mutants, we next tested whether IgGFc-GAIM can alter cellular PrP solubility. We observed significantly reduced amounts of aggregated/insoluble PrP (as assessed by 6D11 mAb) in N2a22LSc cells treated with IgGFc-GAIM compared with cells treated with an IgG control ($p < 0.002$). We next asked if NPT088 dose-dependently altered the propagation of PrP^{Sc}, by comparing PK-digested and undigested lysates from control IgG- and IgGFc-GAIM-treated N2a22LSc cells. We observed a significant dose-dependent decrease in PrP^{Sc} in N2a22LSc treated with IgGFc-GAIM.

Conclusion. We found that IgGFc-GAIM altered PrP solubility and potently inhibited PrP^{Sc} propagation in a dose dependent manner. These findings support the utility of IgGFc-GAIM for modulation of prion disease progression and associated pathologies and are in line with prior findings on the therapeutic potential of NPT molecules for AD and PD. GAIM-based molecules represent a new class of drug candidates for misfolded protein diseases.

PS.38: Exploring the mechanics of prion conversion using RT-QuIC

Bradley R. Groveman and Byron Caughey

Rocky Mountain Labs/NIAID/NIH; Hamilton, MT USA

The pathogenic prion protein (PrP^{Sc} or PrPRes) can induce the conversion of its normal counterpart, PrP^{C} or PrP^{Sen}, into PrP^{Sc} like conformations which assemble into amyloid fibrils. This ability of abnormal forms of prion protein (PrP) to seed the formation of amyloid fibrils from recombinant PrP^{Sen} (rPrP or $rPrP^{Sen}$) has served as the basis for the development of highly sensitive tests for prion diseases. The Real-Time Quaking-Induced Conversion (RT-QuIC) reaction is one such assay which monitors PrP^{Sc}-seeded fibril formation using the fluorescent dye, thioflavin T. This assay allows for the rapid, ultrasensitive detection of PrP^{Sc} in biological samples and has been shown to be the most specific cerebrospinal fluid-based test for CJD diagnosis in humans. RT-QuIC also provides the opportunity to explore the mechanism and dynamics of the prion conversion reaction. We have studied early and intermediate stages of the conversion reaction with respect to kinetics, seed characteristics, inhibitors, potential cofactors, and metal ion interactions. In this study we use the RT-QuIC reaction to investigate the distribution of PrPRes oligomers and fibrils with seeding activity generated at different stages of conversion. These studies can provide important insight into the mechanisms by which seeded conversion of PrP occurs.

PS.39: Chemi-prions that induce cell death

Olivier Julien,[1] Martin Kampmann,[2] Michael C. Bassik,[2] Julie A. Zorn,[3] Jonathan S. Weissman[2] and James A. Wells[2]

[1]University of Alberta; Edmonton, AB Canada; [2]University of California, San Francisco; San Francisco, CA USA; [3]University of California, Berkeley; Berkeley, CA USA

The cellular mechanisms by which prion and prion-like fibrils (e.g., tau, amyloid-β fibrils) lead to neurotoxicity remain largely enigmatic. Additionally, since PrP^{Sc} can be propagated indefinitely in immortalized cultured cells without any major deleterious consequences, unraveling the mechanism of prion neurotoxicity may benefit from alternative approaches.

A high-throughput screen previously performed in our laboratory has successfully identified a series of small-molecule analogs that lead to an activation of the executioner procaspases, providing a direct and rapid way to induce apoptosis in various cancer cell lines.[1] We subsequently found that most of these activators, including the most potent of them called 1541, self-assemble and form homogenous structured fibrils in solution.[2]

We are currently investigating whether there are similarities between the pathways involved in 1541 chemical fibril-induced toxicity and the pathways subverted by proteinaceous fibrils that cause neurodegeneration. We speculate that 1541 may constitute a suitable small chemical molecule mimetic for extracellular proteinceous fibrils and may provide insight into their mechanism of neurotoxicity.

In order to understand how these fibril-forming small-molecules, which we name here "chemi-prions," are able to induce apoptosis in cell culture, we used ultracomplex shRNA libraries (25 shRNA/gene)[3,4] to identify genes that when knocked down would protect or sensitize cells against 1541-induced apoptosis. By analyzing two replicates of a focused screen, we obtained a list of robust candidate genes likely involved in 1541 toxicity. Interestingly, several of them are involved in endocytosis and vesicle trafficking. Using flow cytometry and cell death assays, we have confirmed some of these targets by using single-knockdown stable cell lines, and pharmacological inhibition. Finally, we are using fluorescence microscopy to examine 1541 cellular uptake and trafficking in living cells.

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PS.40: A new computational platform for predictive description of pathological conversion of prion proteins

Andriy Kovalenko,[1] Nikolay Blinov[1,2] and David S. Wishart,[1,2]

[1]National Institute for Nanotechnology; Edmonton, AB Canada; [2]University of Alberta; Edmonton, AB Canada

The ultimate research goal in the area of prion-like diseases is to develop effective tools for diagnostic and therapeutics against the prion and other neurogenerative diseases. Fundamental insight into mechanisms of pathological conversion of the prion protein (and other amyloidogenic proteins and peptides) and the molecular structure of toxic and infectious agents behind these diseases will provide a foundation for identification of biomarkers for diagnostics and rational design of effective antiprion drugs.

In the process of conversion, the prion protein transforms from its soluble normal isoform into insoluble amyloid fibrils.[1-4] Soluble oligomers which may form in the process of conversion (as intermediates or end product of an alternative conversion route) are believed to be neurotoxic agents in many neurodegenerative diseases. Solvation effects play a crucial role at all stages of conversion, from being a driving force behind the initial aggregation of misfolded proteins to contributing to stability of mature amyloid fibrils. Accurate molecular description of solvation effects is essential for predictive modeling of pathological conversion of prion proteins, interpretation of experiment data on prions structure and molecular mechanisms of conversion, and rational design of antiprion compounds.

We propose to include the 3D-RISM-KH molecular theory of solvation[5-7] into computational biology platform for predictive description of the all stages of the pathological conversion of the prion protein. 3D-RISM-KH accurately and computationally efficiently accounts for hydrophobic effects, structural solvation and desolvation in various environments, including crowded inter- and intracellular space. We developed a multiscale coupling of multi-time-step molecular dynamics (MTS-MD) of a biomolecule steered with effective solvation forces obtained from 3D-RISM-KH, and implemented it in the Amber molecular dynamics package.[3] Further, we incorporated 3D-RISM-KH into the protein-ligand docking framework implemented in the AutoDock package, to properly account for molecular specificity and composition of solution species, including solvent, ligand at a finite concentration, buffer, etc.[8,9]

Here, we demonstrate how this new approach can be used to identify binding modes of physiological ligands and antiprion compounds (inhibitors of pathological conversion of amyloidogenic proteins) accounting for both molecular specificity of a ligand and solvent conditions, which allows one to study concentration effects on protein-ligand binding in fragment based rational drug design.[10,11]

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PS.41: A novel protein motif that targets misfolded protein assemblies

Rajaraman Krishnan

NeuroPhage Pharmaceuticals; Cambridge, MA USA

Protein misfolding and amyloid assembly plays a central role in the pathobiology of several neurodegenerative diseases including Alzheimer, Parkinson and prion diseases. Using a variety of biochemical and biophysical methods we have established that purified formulated filamentous bacteriophage M13 binds and disaggregates amyloid-β, tau, yeast and mammalian prions, and α-synuclein in a sequence independent manner. Structural studies on M13 and its variants allowed us to identify a 25 KDa Generic Amyloid Interaction Motif (GAIM) that facilitates this activity with high specificity. GAIM blocks propagation of recombinant prions in protein misfolding cyclic amplification (PMCA) reactions and inhibits oligomer-induced cell toxicity. An immunoglobin (Ig) fusion of GAIM reduces PrP aggregates in cell culture systems and recapitulates all the anti-amyloid activities of the phage. Binding and remodeling activities of GAIM allow efficient sequestering of aggregation-prone edge strands and represents a therapeutic approach for targeting the reduction of misfolded proteins in disease.

PS.42: The influence of strategic D-amino acid substitution on amyloid β 14–23 aggregation

Jitendra Kumar and Valerie Sim

Centre for Prions and Protein Folding Diseases; Edmonton, AB Canada

Alzheimer disease (AD) is the most common neurodegenerative protein folding disease. At the basis of AD is the protein amyloid β (Aβ), whose aggregation is believed to trigger the molecular cascade of AD pathogenesis. Given its long preclinical stage, AD cannot be diagnosed when this cascade first begins, and no disease-modifying treatments are available. Thus, diagnostics and treatments targeting this amyloid cascade are much needed. Requirements for any detection or inhibition molecule include that it be relatively stable, specific, and non-toxic. D-amino acid peptides are less prone to proteolysis, peptide binding specificity can be maintained, and toxicity can be minimized because they introduce only minor chemical modifications. D-amino acid constructs have also been successfully used as inhibitory peptides in the past. We therefore chose D-amino acid substitution as a strategy by which to bind to and interfere with the aggregation-prone region of Aβ.

The Aβ peptide 14–23 is one of the smallest self-assembly sequences within the Aβ peptide. Within this region, residues 17–21 have recently been identified as an important site for self-adhesion. Furthermore, structural data from Aβ fibrils suggest that phenylalanine (Phe) at positions 19 and 20 are part of the amyloid core. Given that benzene rings from Phe 19 and 20 are able to form intermolecular stacks, these may be fundamental to the stability of Aβ fibrils. We hypothesized that D-amino acid substitution of these Phe within the 14–23 peptide might produce a peptide able to: (1) selectively and diagnostically bind the disease-associated forms of Aβ; (2) block Aβ self-assembly; and/or (3) weaken or alter self-assembly, producing non-toxic or easily degraded forms of Aβ aggregate.

We synthesized Aβ 14–23 peptides with D-Phe at substituted at position 19 and/or position 20 using a Liberty1 peptide synthesizer. Peptides were purified using a c-18 column on a Gilson HPLC. Aggregation reactions were performed by incubating full length Aβ peptide (1–42) with the synthesized peptides in PBS at 37°C and monitoring aggregation by Thioflavin T (ThT) fluorescence. Toxicity of the peptides and aggregate species was determined by MTT assay in cell culture. Structural characteristics were determined using light scattering and electron microscopy.

Peptide 14–23 is known to readily form ThT positive aggregates. By substituting residues 19, 20 or both with D-Phe, we abolished this aggregation tendency. In addition, peptides with substitutions at only position 19 significantly altered aggregation tendencies of Abeta (1–42). Toxicity and structural profiles are presented.

PS.43: Cross-species seeding of recombinant prion protein displays species barriers at protein sequence level

Sofie Nyström and Per Hammarström

Linköping University; IFM-Chemistry; Linköping, Sweden

Accumulation of misfolded prion protein (PrP) is the hallmark of transmissible spongiform encephalopathy (TSE) in a range of different mammalian species. The transmissibility and spreading mechanism in vivo likely includes donor-templated misfolding of the host native PrP.[1] Epidemiologic studies and experiments in laboratory animals have demonstrated that there are species barriers between certain species, e.g., suggesting a barrier preventing transmission of TSE from sheep to man, whereas transmission appears to be allowed from cow to man. Experiments performed already in the early days of prion research have shown that adaptation occurs when crossing the species barrier.[2] This process is nowadays called strain adaptation and may allow formation of nascent strains that become transmissible in otherwise non-susceptible hosts. Differences in sequence, conformation, and dynamics of misfolded PrP as well as the native PrP of the host are hence of interest to understand these processes on a molecular level.

Because misfolding of PrP into an aggregated form is a key step in TSE, the misfolding process which ultimately culminates in amyloid fibrils in vitro affords an interesting assay to study cross-seeding. In our lab we have established a kinetic assay protocol for investigating differences in spontaneous and seeded fibrillation kinetics of the human prion protein (HuPrP90–231) under near native conditions (PBS pH 7.3, 37°C, shaking).[3] Recently we extended this protocol to a convenient in situ thioflavin T (ThT) 96-well format.[4] We hereby recently demonstrated dramatic differences in HuPrP90–231 fibril formation propensity as a consequence of single substitutions in position 129.[4] In the current project we aim to decipher the molecular basis for species barriers by cross-seeding recombinant PrP from a number of different species using this fibrillation assay. The cross-seeding study includes full length protein of human, bovine, porcine, canine, feline, and murine PrP.

Our results demonstrate that all the investigated proteins can form amyloid fibrils in the assay, but with different efficiency. Self-seeding and interspecies cross-seeding implicate that there is a species barrier at the protein misfolding level leading to variable lag times dependent on both the native protein sequence and the seed. We hence foresee that these experiments can form a molecular basis for seeding and cross-seeding efficiency for understanding species barriers and strain adaptation in TSEs.

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PS.44: The dynamic structure of Syrian hamster prion protein β-intermediates

Carlene S. Starck,[1] Karen Simonetti,[1] Patrick Walsh,[2, 1] Sacha Larda,[3] Sameer Al-Abdul-Wahid,[3] Scott Prosser[3] and Simon Sharpe[1]

[1]The Hospital for Sick Children; Toronto, ON Canada; [2]Department of Biochemistry; University of Toronto; Toronto, ON Canada; [3]Department of Chemistry; University of Toronto; Toronto, ON Canada

Prion disease susceptibility has been shown to correlate with the propensity of the natuve state of the mammalian prion protein (PrP^C) to undergo conversion to predominantly β-structured, non-fibrillar misfolding intermediates. either direct.[1] These β-state intermediates are anticipated to play an important role in prion disease pathology, during infection with PrP^{Sc} and in cell death during neurodegeneration, however little is known about their molecular structure. Therefore, obtaining a structural model for these oligomeric intermediates will provide a better understanding of PrP misfolding pathways, define the relationship of the β-state with PrP^{Sc} and assist in determining the role of oligomeric PrP in the pathogenesis of prion diseases.

We are using a NMR spectroscopy and other biophysical tools to obtain a detailed characterization of the molecular structure and dynamic behavior of stable β-state oligomers formed during misfolding of the Syrian hamster prion protein (ShaPrP),[1] and have recently presented evidence that these intermediates are octameric, with a tetramer of dimers morphology. The structurally ordered core is composed of intermolecular in-register parallel β-sheets, suggestive of the strand arrangement observed in previous studies of amyloid fibrils formed by PrP.[2,3] Based on our current experimental data, we propose a direct helix to sheet transition concurrent with oligomerization of the β-state.

Additionally, we have elucidated details of the dynamic equilibrium between non-native monomers, octamers and higher-order oligomers coexisting in the β-state. 19F NMR of β-state PrP enriched with 3-fluoro-phenylalanine revealed the adoption of three spectroscopically resolved states in equilibrium, and allows us to examine the thermodynamic parameters driving PrP misfolding and aggregation. Taken together, our data provide new insight into molecular rearrangements that occur during PrP misfolding.

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PS.45: Essential collective dynamics: A novel structural biology framework to discover molecular mechanisms of protein misfolding

Maria Stepanova

National Research Council of Canada; University of Alberta; Edmonton, AB Canada

Protein misfolding diseases are a growing group of neurodegenerative disorders related to improper folding of proteins and formation of amyloid fibrils accumulating in the brain and other tissues. Presently, more than 20 proteins have been associated with various misfolding disorders, such as prion proteins with the transmissible spongiform encephalophaties, superoxide dismutase 1 with amyotrophic lateral sclerosis, amyloid-β peptide with Alzheimer disease, or α-synuclein with Parkinson disease. Morphological similarities among some amyloid aggregates from different proteins suggest that the mechanisms of the conversion may be related, however no obvious similarities in sizes, associations with particular aminoacid sequences, or tertiary structure motifs could be found. For protein misfolding diseases could be efficiently cured, a detailed understanding of amyloidigenic conversion pathways and possible intermediate structures is required. This in turn requires a development of dedicated computational methods to identify and characterize in silico transient misfolding intermediates. This presentation overviews a novel computational framework, which we recently introduced[1-4] and applied to characterize the dynamics of prion proteins (PrPs) monomers and dimers.[3-6] The underlying method, which we denote as the essential collective dynamics (ECD) method, allows one to identify persistent dynamic correlations of atomic motions from short (~0.2 ns) molecular dynamics trajectories. The method relies on a fundamental statistical-mechanical concept,[1,2] which makes the outcomes rigorously interpretable and directly comparable with NMR structural data representing longer timescales.[1-5] Employing the ECD method, we were able to identify dynamical "signatures" of relative conversion resistance in PrP constructs.[5,6] Furthermore, the method has allowed us to find new evidence about the potential molecular mechanism of prion conversion.[6] Using this framework, we also created a novel simulation tool that can efficiently model conformational fluctuations in proteins over millisecond timescales. In this presentation examples will be shown, how the ECD structural descriptors such as dynamic domains of correlated motion, main-chain flexibility, and side chain networks could be employed to characterize the dynamical stability of proteins and their complexes, and discuss capabilities of the method to capture early stages of protein misfolding.

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PS.46: The crystal structure of an octapeptide repeat of the mouse prion protein in complex with the POM2 Fab fragment

Mridula Swayampakula,[1] Pravas K. Baral,[1] Adriano Aguzzi,[2] Nat Kav[1] and Michael James[1]

[1]University of Alberta; Edmonton, AB Canada; [2]University Hospital Zurich; Zurich, Switzerland

Transmissible Spongiform Encephalopathies (TSEs) are a class of fatal, infectious neurodegenerative disorders. Scrapie in sheep, Bovine Spongiform Encephalopathy (BSE) in cattle, Chronic wasting disease(CWD) in deer and elk are some of the TSEs affecting animals and Kuru, Creutzfeldt-Jakob disease (CJD), Fatal Familial Insomnia (FFI) are some of the TSEs affecting humans. The pathogenesis occurs due to the autocatalytic conformational conversion of the cellular prion protein (PrP^{c}) into a β sheet-rich, infectious conformation (PrP^{Sc}). Recombinant PrP has a structured C-terminal domain and a highly flexible N-terminal domain. The flexible N-terminal domain consists of 4–5 repeats of an unusual glycine rich eight amino acids long peptide known as the 'octapeptide repeat' domain (OR). Our objective is to study the structural characteristics of the OR domain using X-ray crystallography. It is common practice to use the Fab domains of antibodies for promoting crystallization of difficult proteins. Hence, we chose to use the Fab fragment of the monoclonal POM2 IgG as it has its epitope in the OR domain.

We successfully crystallized the tandem OR repeats of mouse PrP in complex with the POM2 Fab fragment. We report the first crystal structure of an octapeptide repeat peptide bound to the POM2 Fab antibody fragment. The structure was solved at a resolution of 2.3 Angstroms by molecular replacement and the asymmetric unit contains two 1:1 complexes of the POM2 Fab-OR2 complex. While several studies have previously predicted a β-turn like structure of the unbound octapeptide repeats, our structure shows an extended conformation of the octapeptide repeat when bound to a molecule of the POM2 Fab indicating that the bound Fab disrupts any putative native β-turn conformation of the octapeptide repeat.

PS.47: Prion mutability during in vitro replication by protein misfolding cyclic amplification (PMCA)

Ilaria Vanni, Sergio Migliore, Gian Mario Cosseddu, Michele A. Di Bari, Laura Pirisinu, Claudia D'Agostino, Stefano Marcon, Geraldina Riccardi, Umberto Agrimi and Romolo Nonno

Department of Food Safety and Veterinary Public Health; Istituto Superiore di Sanità; Rome, Italy

We have recently shown that PMCA using vole brain homogenates as substrate is highly efficient for both homologous and heterologous PrP^{Sc} amplification.[1] With the aim to investigate the sensitivity of detection of natural scrapie by vole-PMCA, we seeded PMCA reactions with serial 10-fold dilutions of sheep scrapie isolates and performed multiple PMCA rounds. After 7–8 PMCA rounds, sheep PrP^{Sc} was amplified from 10^{-5}- to-10^{-6} dilutions. In most cases, PMCA-derived PK-treated PrP^{Sc} (PrP^{res}) showed an electrophoretic pattern (21K) indistinguishable from the original sheep PrP^{res}. However one sheep isolate, SS21, repeatedly induced the emergence of an alternative PrP^{res} conformer, characterized by a 14K, glycosylated C-terminal fragment. This "mutant" PrP^{res} emerged exclusively from reactions seeded with the highest dilutions (10^{-4}-to-10^{-6}) of SS21. Further studies by serial PMCA showed that the emerged mutant, named 14K, was autocatalytic in vitro, being able to propagate stably, faithfully and as efficiently as 21K. We next investigated the biological properties of PMCA-derived 21K and 14K by vole bioassay, in comparison to direct transmission of sheep SS21. These studies showed that: (1) SS21 induced exclusively the deposition of 21K PrP^{res} in voles; (2) PMCA-derived 21K was infectious and induced a phenotype indistinguishable from vole-adapted SS21; (3) PMCA-derived 14K was not infectious. Thus, we have serendipitously observed the emergence in vitro of a defective PrP^{Sc} species, able to propagate in vitro but not in vivo. The finding that this phenomenon was exclusively observed in PMCA reactions seeded with highly diluted inocula strongly suggests that it did not result from the selective amplification of a minor PrP^{Sc} species present in the SS21 sheep isolate. Alternatively, under high dilution amplification, SS21 could have undergone a molecular switch or mutation, resulting in a defective prion mutant, as reported for quasispecies viral populations replicated at low multiplicity of infection (MOI). We next tested this hypothesis in a series of homologous PMCA studies using vole-adapted SS21 and PMCA-derived 21K in 2 different experimental settings: high MOI and low MOI serial PMCA. Preliminary data show that 14K didn't emerge from homologous PMCA, although we had evidence of other variations in the electrophoretic patterns of PrP^{res}. Overall, our findings indicate that PrP^{Sc} conformational variants can emerge during in vitro replication of prions and that PMCA could be a powerful tool to investigate the phenomenon of prion strain mutation.

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PS.48: Heparin modulation of prion seeding activity

Tuane C. Vieira,[1] Byron Caughey[2] and Jerson L. Silva[1]

[1]Federal University of Rio de Janeiro; Rio de Janeiro, RJ, Brazil; [2]National Institutes of Health; Hamilton, MT, USA

Keywords: prion, gycosaminoglycan, aggregation, neurodegeneration

Introduction. The conversion of PrP into scrapie PrP is the central event of prion diseases (TSEs). Such conversion and propagation is seeded or templated by a polymerization mechanism. Some authors have suggested that glycosaminoglycans (GAGs) directly convert PrP into a protease resistant form, while others have proposed that these molecules have a protective activity. Our group recently reported that low molecular weight heparin (LMWHep) does not induce recombinant mouse prion protein (rPrP23–231) conversion, protecting rPrP23–231 from RNA-induced aggregation.[1]

Materials and Methods. Real-time quaking-induced conversion (RT-QuIC) is an assay in which disease-associated PrP initiates a rapid conformational transition in recombinant PrP, resulting in the formation of amyloid fibrils that can be monitored in real time using the dye thioflavin T. We used rPrP from mouse and hamster as substrate (23–231 and 90–231), and TSE-associated forms were from mouse and hamster brain homogenates (RML and 263K strain respectively). LMWHep was used in order to determine the effect of this GAG on PrP fibrillization.

Results and Discussion. In the present work, we show that LMWHep delays and decrease fibril formation. It also inhibits fibrilization depending on the seed used. There is no effect when rPrP 90–231 is used, or with high salt concentration. Moreover, it is effective when added at the lag phase of the polymerization process. When a soluble LMWHep-rPrP complex is added to the reaction, no fibrils are detected. On the contrary, the addition of a LMWHep-rPrP aggregated complex results in conversion.

Conclusion. Through electrostatic interactions, LMWHep interaction with PrP N-terminal domain, modulates PrP fibrilization. It affects the nucleation processes and the formation of oligomers, the first step of fibrilization. Our findings may explain the protective effect of these molecules in different models.

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PS.49: Shaking alone converts PrP^c to β-sheet rich fibrils

Carol L. Ladner[1] and David S. Wishart[1,2]

[1]University of Alberta; Edmonton, AB Canada; [2]National Institute for Nanotechnology; Edmonton, AB Canada

The formation of β-sheet rich PrP oligomers and fibrils from PrP^c is thought to be a key step in the development of prion diseases. Various methods are available to convert recombinant PrP^c into β-sheet rich fibrils using denaturing conditions, phospholipids or mildly acidic pH (pH 4). All of these methods also require shaking, quaking or sonication. We have identified that shaking alone and sonication alone causes conversion of recombinant PrP^c(Syrian hamster PrP90–232) to β-sheet rich oligomers and fibrils at near physiological pH (pH 5.5 to pH 6.2). Furthermore, this conversion does not occur when the water-air interface is eliminated in the shaken sample. We have analyzed this conversion using circular dichroism, resolution enhanced native acidic gel electrophoresis (RENAGE), and electron microscopy. Using RENAGE we also see that shaking alone induces oligomerization for full-length PrP (mouse PrP 23–231) and the C-terminal PrP domain (mouse PrP 120–231). In addition, we have compared the conversion efficiency using RENAGE by shaking alone of recombinant PrP constructs of hamster, mouse and cervids (white-tailed deer, mule deer and elk). We propose that this method will provide a means to test species susceptibility and the effectiveness of anti-prion small molecules using physiological conditions.

PS.50: Phospholipid induced PrP intermediate exhibits accessibility to the polybasic binding domain

Ashley A. Zurawel, Daniel J. Walsh, Sean F. Fortier and Surachai Supattapone

Department of Biochemistry; Geisel School of Medicine at Dartmouth; Hanover, NH, USA

Delineating the process whereby PrP^{Sc} recruits the normal cellular isoform PrP^C is necessary to develop a tractable mechanism for prion propagation. Addressing this biological question has been greatly aided by the development of in vivo and in vitro models, such as the ability to generate infectious recombinant prions.[1] We report here that the combination of recombinant PrP^C protein and phosphatidylglycerol, integral components of in vitro generated prions, produces a conformation that like PrP^{Sc}, which specifically interacts with the N-terminal polybasic domain of PrP^C. This polybasic domain has previously been shown to selectively bind PrP^{Sc}, and is required to support the propagation of recombinant PrP^{Sc} in vitro.[4] This suggests an essential role for lipid-protein interaction in conversion and provides a valuable intermediate species well suited for biochemical study. We characterize its ultrastructure using electron microscopy, and,

additionally, we use a photoactivatable version of the polybasic peptide to specifically label the PrP intermediate. This PrP-lipid intermediate presents a novel way to gain insight into the PrP^{C}-PrP^{Sc} replicative interface, and contributes to our understanding of prion pathogenesis.

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PS.51: Enzymatic activity of a subtilisin homolog, Tk-SP, from thermococcus kodakarensis in detergents and its ability to degrade the abnormal prion protein

Kazufumi Takano

Kyoto Prefectural University; Kyoto, Japan

Background. Proteases form a major part of the industrial enzyme market, and are applied to detergents as well as food, leather, and fabric processing, and are also used as catalysts in organic synthesis and as therapeutics. Incorporation of proteases into detergent is essential to obtain certain characteristics, for example, activity and stability of the compounds used in detergent, such as surfactants. Tk-SP is a member of subtilisin-like serine proteases from a hyperthermophilic archaeon Thermococcus kodakarensis. It has been known that the hyper-stable protease, Tk-SP, could exhibit enzymatic activity even at high temperature and in the presence of chemical denaturants. In this work, the enzymatic activity of Tk-SP was measured in the presence of surfactants (four nonionic detergents, three anionic detergents, two cationic detergents, and three amphionic detergents) and EDTA at high temperatures. In addition, we focused to demonstrate that Tk-SP could degrade the abnormal prion protein (PrP^{Sc}), a protease-resistant isoform of normal prion protein (PrP^{C}).

Results. Tk-SP was observed to maintain its proteolytic activity with surfactants and EDTA at 80 and 90℃. Furthermore, Tk-SP was found to be highly stable in the presence of both 0.1 and 1% (w/v) nonionic surfactants. Particularly, Tk-SP retained more than 100% of its activity in the presence of four of the nonionic surfactants, namely, EMULGEN 147, EMULGEN LS-114, EMULGEN PP-290, and RHEODOL Tw-0120V. Tk-SP retained 80% of its activity in the presence of 0.01% (w/v) EDTA, suggesting that this enzyme will be effective in the presence of 0.01% (w/v) EDTA, which is a commonly used concentration of EDTA in detergents. We optimized the condition in which Tk-SP functions efficiently, and demonstrated that the enzyme is highly stable in the presence of 0.05% (w/v) nonionic surfactants and 0.01% (w/v) EDTA, retaining up to 80% of its activity. Additionally, we also found that Tk-SP can degrade PrP^{Sc} to a level undetectable by western-blot analysis. A combination of 0.02 mg/ml (0.4 mM) Tk-SP and 1% (w/v) SDS was noted to digest infectious MBH, suggesting that Tk-SP has the ability to digest PrP^{Sc} in the absence or presence of SDS.

Conclusion. Our results indicate that Tk-SP showed inherent stability in the presence of both surfactants and EDTA without site-directed mutagenesis or protein engineering. This means that the enzyme has a great potential for technological applications, such as thermo-stable detergent additives. In addition, it is also suggested that Tk-SP-containing detergents can be developed to decrease the secondary infection risks of transmissible spongiform encephalopathies (TSE).

PS.52: Validation of differentially expressed proteins in scrapie-infected mice plasma using multiple reaction monitoring assays

Sang Ho Choi, Su Yeon Kim, Chi-Kyeong Kim, Bo Yeong Choi, Jeongmin Lee, Kyu Jam Hwang and Young Ran Ju

National Institute of Health; KoreaCDC; Cheongwongun, Chungcheongbukdo, Korea

Creutzfeldt-Jakob disease (CJD) is one of the fatal neurodegenerative diseases in human prion disease known as transmissible spongiform encephalopathies (TSEs) caused by infectious prion proteins. The definite CJD has been diagnosed through the detection of PrP^{Sc} pathogen in brain or tonsil tissues, and that is only post-mortem diagnosis. However, it is hard to perform the diagnosis using patients' tissues in Korea that has the traditional culture be reluctant damaging their family bodies like autopsy. We performed validation for several potential protein markers identified in body fluid to develop the effective diagnostic markers in early or late infectious stages. The several protein markers, including 14-3-3, S-100, neuron-specific enolase (NSE), and tau proteins have been reported to useful for CJD diagnosis with body fluid.

Accurate quantification for potential proteins discovered in plasma of scrapie infected mice was performed to time courses (50 dpi, 120 dpi, 171 dpi) by using proteomic tools and multiple reaction monitoring (MRM). Twelve of proteins in 50 dpi, 11 of proteins in 120 dpi, 8 of proteins in 171 dpi and 1 of proteins in both 50 and 120 dpi were validated as significantly upregulated proteins. Especially, fibrinogen and myoglobin were enormously upregulated compare with control in early stage. Biological process clustering of more than 1.5 fold increased proteins was performed by Clue Go. These proteins were mainly associated with classical pathway of complement activation, wound healing and response to cytokine stimulus pathway.

We consider that peptide MRM assay is a rapid and specific assay platform for biomarker validation to supplement significant problem of specific antibody production of capture assay verification. We also expect that validated proteins would be applied

to diagnosis of antemortem for suspected CJD as surrogate biomarkers with high specificity and sensitivity through the effectiveness assessment of expression level or in vivo approach.

PS.53: Humic substances underlies the odds of environmental TSEs transmission

Gabriele Giachin,[1] Joanna Narkiewicz,[1] Scaini Denis,[2] Rinki Saha,[1] Alja Margon,[3] Liviana Leita[3] and Giuseppe Legname[1]

[1]Laboratory of Prion Biology; Department of Neuroscience; Scuola Internazionale Superiore di Studi Avanzati (SISSA); Trieste, Italy; [2]Life Science Department; Center for Neuroscience B.R.A.I.N.; University of Trieste; Trieste, Italy; [3]Consiglio per la Ricerca e Sperimentazione in Agricoltura (CRA); Gorizia; Italy; Gorizia, Italy

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders caused by the misfolding of the cellular form of the prion protein, PrP^{C}, into its β-sheet-rich isoform, PrP^{Sc}. Scrapie and chronic wasting disease are the only TSEs that appear to be environmentally transmissible. Increasing evidence suggests that soil may serve as a natural reservoir of prion infectivity. Attachment to soil particles is likely to influence the persistence and infectivity of prions in the environment. Soil with high clay content relative to organic matter may enhance prion transmission. The contribution of natural soil organic matter in prion adsorption has been neglected. Here, we present evidence of the interaction between humic substances (HS) with both recombinant mouse (Mo) PrP and RML prion strain. Natural HS interact with MoPrP forming insoluble adducts. Biophysical and atomic force microscopy imaging experiments showed that MoPrP retains its native folding when it is encapsulated in ordered structures made of HS. The PrP-HS complex is protected from protease-K and from ovine ruminal digestion. Interestingly, HS induced elimination of prions from chronically scrapie-infected mouse hypothalamic cells, ScGT1, in a dose-dependent manner. Prion encapsulation in HS may be highly environmentally relevant for soil rich in organic matter. Prions should be strongly retained in soils with a higher organic matter content, which would thus reduce the odds of infectivity among grazing lands. The anti-prion activity of HS might have an impact in reducing prion bioavailability in the intestinal tract of free-ranging animals, and in preventing the environmental transmission of TSEs among ruminants.

PS.54: Heparan sulfate levels reduced in PrP knockout mice

S. David Cullis-Hill, Wendy S. Pratt and John F. McEwan

Sylvan Pharmaceuticals; Sydney, NSW Australia

Introduction. Prion protein has been extensively researched over many years; however, the biological functions of prion protein remain elusive. Many relationships between PrP and heparan sulfate proteoglycans (HSPGs) are known. Heparan sulfate (HS) is involved in diverse biological processes regulated through various HS-binding proteins, including growth factors. Disruption of glycosaminoglycan metabolism has been demonstrated in prion disease, along with changes in transcription patterns for HSPG synthesis in prion infected cells, suggesting a link between HS and PrP^{Sc} infectivity. Heparan sulfate containing the 10E4 antigen is associated with scrapie lesions. Glypican-1, bearing HS chains, is involved in PrP cycling from the cell surface. Here, additional evidence shows a reduction in HS levels in PrP-knockout mice, as measured by several independent techniques.

Materials and Methods. HS levels were compared in wildtype and transgenic PrP-knockout mice. Mice were perfused with PBS and organs harvested then frozen until required. Tissue was homogenized, treated with pronase and extracted with Trizol. The dialysed extract was subsequently treated with chondroitinase ABC, neuraminidase and benzonase, before isolation of the purified HS by DEAE chromatography. HS analysis was performed with agarose gel electrophoresis, dimethylmethylene blue assay and HPLC.

Results. HS levels in multiple organs (brain, kidney and liver) were shown to be lower in PrP-knockout mice compared with wildtype animals. Analysis of the disaccharide composition of both groups of HS afforded no significant difference in the major disaccharide composition—although the concentration was lower in knockout animals in accordance with the decrease in total concentration observed. Changes in domain structure and periodicity in the HS are expected to be subtle and minor variations in the lesser sulfated regions were noticed, however these species are yet to be identified.

Discussion. Reduced HS levels were observed in PrP knockout mice using three independent techniques. These results suggest that PrP is involved in the regulation of synthesis of HS or HSPG expression and/or signaling. This data may suggest HS is implicated in many of the complex and diverse interactions of PrP with other proteins: such a relationship could explain phenotype in PrP knockout animals.

PS.55: Starved for iron: Imaging of prion-infected brain reveals localization and chemical speciation of sequestered iron

M. Jake Pushie,[1] Kurt H. Nienaber,[1] Holger Wille,[2] Jan Stoehr[3] and Graham N. George[1]

[1]University of Saskatchewan; Saskatoon, SK Canada; [2]University of Alberta; Edmonton, SK Canada; [3]University of California; San Francisco; San Francisco, CA USA

The prion protein (PrP) is expressed in many mammalian tissues, with the highest level of expression found in the brain and central nervous system. This protein plays roles in neuronal development, maintenance of synaptic structure and function and maintaining long-term potentiation in the hippocampus. Pushie and colleagues have also shown that PrP expression correlates

with the amount of iron, copper and zinc within specific brain regions from transgenic mice.[1]

Prion diseases comprise a family of closely related neurodegenerative diseases which arise, in part, through a structural change in PrP, which results in misfolding and self-assembly into neurotoxic oligomers and aggregates within the brain. There are a number of human forms of the disease (named according to their presenting clinical signs), and prion disease has been documented in a range of other mammals, including scrapie in sheep, bovine spongiform encephalopathy in cattle, and chronic wasting disease in several species of cervid, as examples. Prion disease is ultimately fatal and there is currently no cure, nor any treatment options.

Using a mouse model of prion disease (the RML strain of mouse-adapted scrapie) in an inbred strain of mice (FVB) it has previously been shown that the infectious prion material (termed PrP^{Sc}) accumulates throughout the brain. Ferritin, an iron storage protein, is advantageously co-isolated with PrP^{Sc} from infected brain homogenate, and recent work by Singh, et al.[2] has shown that the prion-infected brain presents a phenotype of iron deficiency, despite an otherwise sufficient level of iron being present in the brain. Despite the presence of the ferritin protein which is co-isolated with PrP^{Sc} from brain homogenate, detection of the protein itself is not ideal for direct quantitative measurement of iron within biological tissues. Using synchrotron-based X-ray fluorescence mapping we demonstrate that iron is highly accumulated in particular fiber tract regions in the RML-infected FVB mouse brain. We have also employed X-ray absorption spectroscopy to characterize the chemical form of the accumulated iron in situ, which confirms that much of this iron is bound to ferritin. These results provide critical new data on the brain's response to prion infection, further clues to the apparent iron deficiency in prion disease and potential new areas of investigation.

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PS.56: Penetrant null alleles of the *Sprn* gene do not produce embryonic lethality in combination with PrP^{C}-deficiency

Nathalie Daude,[1] Serene Wohlgemuth,[1] Inyoul Lee,[2] Rose Pitstick,[3] Hristina Gapeshina,[1] Leroy Hood,[2] George A. Carlson[3] and David Westaway[1,4]

[1]Centre for Prions and Protein Folding Diseases; University of Alberta; Edmonton, AB Canada; [2]Institute for Systems Biology, Seattle, WA USA; [3]McLaughin Research Institute; Great Falls, MT USA; [4]Department of Biochemistry; University of Alberta; Edmonton, AB Canada

The *Sprn* gene encodes Shadoo (Sho), a central nervous system (CNS) glycoprotein with biochemical properties similar to the unstructured region of PrP^{C}, and a candidate for the hypothetical ω protein that maintains PrP^{C} functions in $Prnp^{0/0}$ mice. To understand these relationships better we probed the cell biology of Sho and created knockout mice. Besides full-length and C1 C-terminal fragment, we report here that Sho also produces a 6kDa N-terminal neuropeptide, "N1," which is present in membrane-enriched subcellular fractions of wt and Tg.*Sprn* mice. In genetic studies *Sprn* null alleles were produced that delete all protein coding sequences yet spare the Mtg1 gene transcription unit that overlaps the *Sprn* 3′ untranslated region (3′ UTR). The resulting mice bred to homozygosity were both viable and fertile, and had no overt perturbations in the expression of genes located immediately 5′ and 3′. However, $Sprn^{0/0}$ mice maintained in two distinct genetic backgrounds weighed less than wt mice. Contrasting with lethality reported for *Sprn* knockdown in $Prnp^{0/0}$ embryos using lentiviruses targeted against the Sprn 3′ UTR,[1] we established that double knockout $Sprn^{0/0}$ + $Prnp^{0/0}$ mice deficient for CNS expression of Sho and PrP^{C} are fertile, and viable up to 500 d of age. While divergent results likely reflect the alternative strategies of targeting the *Sprn* 3′ UTR rather than ablating the coding region, our data with penetrant Sprn null alleles rigorously exclude a hypothesis wherein expression of both PrP^{C} and Sho is required for completion of embryogenesis. Rather, in accord with some reports for PrP^{C}, Sho may contribute to trophic pathways that are active postnatally.

References

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